Phenotype and function of B cells in children infected with human immunodeficiency vius

Thesis

PHENOTYPE AND FUNCTION OF B CELLS IN CHILDREN INFECTED WITH HUMAN IMMUNODEFICIENCY VIRUS

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ADCC	Antibody Dependent Cellular Cytotoxicity
AI	Avidity Index
AIDS	Acquired ImmunoDeficency Syndrome
ASC	Antibody Secreting Cells
AU	Arbitrary Units
BAFF	B-cell Activating Factor
BCR	B-cell Receptor
BSA	Bovine Serum Albumin
CCRC	Comprehensive Care and Research Clinic
CD	Cluster of Differentiation
CD4bs	CD4 binding site
CFSE	CarboxyFluorescein Succinimidyl Ester
CNS	Central Nervous System
CSR	Class-Switch Recombination
DC-SIGN	Dendritic Cell-Specific ICAM-3-Grabbing Non-integrin
DMSO	DiMethyl SulfOxide
DNA	Deoxyribonucleic Acid
DT	Diphtheria Toxoid
DTaP	Diphtheria Tetanus and acellular Pertussis vaccine
DTP	Diphtheria Tetanus and Pertussis vaccine
EDTA	Ethylene Diamine Tetraacetic Acid
ELISA	Enzyme Linked ImmunoSorbent Assay
ELISpot	Enzyme Linked Immunospot Assay
ERC	Ethical Review Committee
GALT	Gut Associated Lymphoid Tissue

HAART	Highly Active Anti-retroviral Therapy
HB	Hepatitis B vaccine
HiB	*Hemophilas influenza* type B vaccine
HIV	Human Immunodeficiency virus
HLA	Human Leukocyte Antigen
IPD	Invasive Pneumococcal Disease
IQR	InterQuartile Range
KDH	Kilifi District Hospital
KEMRI	Kenya Medical Research Institute
LTNP	Long Term Non-Progressors
MHC	Major Histocompatibility Complex
mHSA	methylated Human Serum Albumin
MSM	Men who have sex with men
NFκB	Nuclear Factor kappa B
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitors
NRTI	Nucleoside Reverse Transcriptase Inhibitors
OPA	OpsonoPhagocytic Assay
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCV-7	Heptavalent Pneumococcal Conjugate Vaccine
PHiD-CV	Pneumococcal non-typeable *Haemophilus Influenza* protein D conjugate vaccine
PMTCT	Prevention of Mother To Child transmission
PPS	Pneumococcal capsular PolySaccharides
PRISM	PHiD-CV Reactogenicity and Immunogenicity Study in Malindi

PS	Pneumococcal Serotype
RNA	Ribonucleic Acid
SHM	Somatic HyperMutation
SIV	Simian Immunodeficiency Virus
SSC	Scientific Steering Committee
TLR	Toll Like Receptors
TT	Tetanus Toxoid
WHO	World Health Organisation

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Abstract

HIV infection has been shown to affect all lymphocyte populations, including the B-cell compartment. Defects in phenotype and function of B cells have been well characterized in HIV-infected adults. However, only a few studies have investigated the effect of HIV on the B-cell compartment in children, and such studies have been less detailed than in adults. As a result, much remains to be described with regard to B cells in HIV-infected children.

In this study, I assessed the phenotype and function of B cells in HIV-infected children. I also assessed the in vitro effect of various B-cell stimulant-combinations and the effect of recombinant HIV-1 nef protein on the resultant B-cell responses.

The phenotypic defects observed in the HIV-infected children were broadly similar to those observed in HIV-infected adults but with minor differences. Furthermore, high viraemia and low $CD4^+$ T-cell percentages interfered with the age-related accumulation of B-cell memory, suggesting that children of all ages might benefit from immediate initiation of HAART upon diagnosis with HIV-infection. HIV infection was also associated with poor B-cell responses to vaccine antigens, implying that such children may require revaccination upon initiation of HAART. In the in vitro experiments, different B-cell stimulants elicited synergistic effects on B cells. However, HIV-1 Nef did not affect various B-cell responses upon exposure to the B-cell stimulants.

WHO has recently revised the recommendations for management of paediatric HIV. All children below five years of age are to be started on HAART the moment they are diagnosed with HIV, but such recommendations are yet to be adopted into national guidelines. The possibilities of revaccinating HIV-infected children once on HAART need to be evaluated. Even though some studies have reported on the possible causes of B cell defects in HIV, more research needs to be done to elucidate other causes that may offer intervention targets.

Chapter 1 Introduction

1.1 Classification of HIV

Human Immunodeficiency Virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS). It exists in the form of two related viruses, HIV-1 and HIV-2 [1-3]. HIV-1 can be further subdivided into four groups; M ("Main"), O ("Outlier"), N and P, with group M being the most prevalent worldwide. Group M exists in at least 9 genetically distinct clades (subtypes): A, B, C, D, F, G, H, J and K. Occasionally, recombination events between different clades give rise to hybrid forms e.g. A/B. Similarly, HIV-2 is divided into 8 groups: A, B, C, D, E, F, G and H. Groups A and B are the only epidemic forms while the others are though to be dead-end infections in humans [4].

1.2 Origins of HIV

HIV-1 and HIV-2 are cross-species (zoonosis) diseases that resulted from crossover of Simian Immunodeficiency Viruses (SIV) from non-human primates. HIV-1 is suspected to have originated from common chimpanzee (*Pan troglodytes*) that naturally host SIVcpz while HIV-2 came from the sooty mangabey *(Cercocebus atys)* that host SIVsm. Evidence to suggest this zoonosis origin include:

1. Phylogenetic relatedness of the SIVcpz and SIVsm with HIV-1 and HIV-2 respectively [5-7].
2. Similarities in genomic organization [5, 6].
3. Geographical overlap between the natural epidemiology of the SIVsm with HIV-2 and SIVcpz with the suspected original epidemic of HIV-1 [8, 9].
4. Existence of SIVcpz and SIVsm in their natural hosts i.e *Pan troglodytes* and *Cercocebus atys* respectively [8, 9].

5. Close contact between the non-human primates and humans as these primates are hunted by humans for food or kept as pets [10, 11].

Some additional evidence suggests that HIV-1 group O may have originated from gorillas [12].

The existence of HIV-1 and HIV-2 in several groups suggests that there were multiple crossover events for each virus i.e. at least 4 events for HIV-1 and 8 events for HIV-2. Indeed, cross-species transmission of SIV has been documented in non-human primates, the most notable being the crossing of SIVsm from captive sooty mangabeys to captive rhesus macaques that gave rise to SIVmac [13-15]. Using molecular clocks, the crossover event has been estimated to have taken place in 1931 (1915-1941) for HIV-1 group M [16]. This estimate is in line with the detection of HIV-1 in samples dating as far back as 1959 despite the fact that the epidemic was only recognized in 1980 [17, 18]. Another study has estimated the introduction of HIV-2 into the human population to have taken place in 1940 (+/-16 years) for group A and 1945 (+/- 14 years) for group B [19].

Due to the limited prevalence of HIV-2 in Kenya, this thesis is written in the context of HIV-1 infections. Therefore, all subsequent discussions are centered on HIV-1.

1.3 Biology of HIV-1

HIV-1 is a ribonucleic acid (RNA) virus with a genome consisting of two positive-sense RNA molecules. It has nine genes that encode for a total of 15 proteins [20]. Three of the nine genes i.e. *gag, pol* and *env* are the major genes and encode for structural proteins and enzymes. They give rise to Gag, Gag-Pol and Env polyproteins respectively. Gag is processed further to give the matrix, capsid, nucleocapsid and p6 protein in addition to two spacer peptides. Gag-Pol gives rise to integrase, reverse

transcriptase and protease while the Env gives rise to the surface gp120 and trans-membrane gp41. The remaining six genes encode four accessory proteins (Nef, Vpu, Vpr and Vif) and two regulatory proteins (Tat and Rev) that influence various aspects of immune evasion or facilitate viral replication [20]. Of particular interest in one chapter of this thesis is Nef (negative factor), which has been shown to have a wide range of interactions with the host's immune cells. It activates $CD4^+$ T cells and monocytes, rendering them more permissive to infection and viral replication [21, 22]. It also reduces expression of MHC class I on infected cells to protect them from being killed [23] in addition to down-regulating CD4 expression on helper T cells to enhance the budding off of newly formed virions [24]. It has also been shown to inhibit antibody class switching to prevent optimal antibody response against HIV [25]. Vpu (unknown virus protein) too has the ability to modulate expression of CD4 on helper T cells by inducing CD4 degradation in the endoplasmic reticulum [26]. It also inhibits the effects of CD317, a tetherin that is a potent HIV restriction factors. CD317 causes retention of formed virions on the surface of infected cells [27]. Vpr (viral protein R) reportedly induces apoptosis of T cells, thus contributing to the depletion of $CD4^+$ T cells [28]. It also causes cell cycle arrest at G2 phase, providing a conducive condition for transcription of HIV genes since HIV-1 LTR appears to be most active at the G2 phase [29]. Vif (virion infectivity factor) induces the degradation of APOBEC3G, a potent HIV restriction factor [30]. Tat (trans-activator of transcription) controls the initiation of transcription and enhances the processivity of the polymerases that are involved in transcribing HIV proteins [31]. Rev (regulator of virion expression) controls the export of viral mRNA from the nucleus to the cytoplasm where HIV proteins are translated [32].

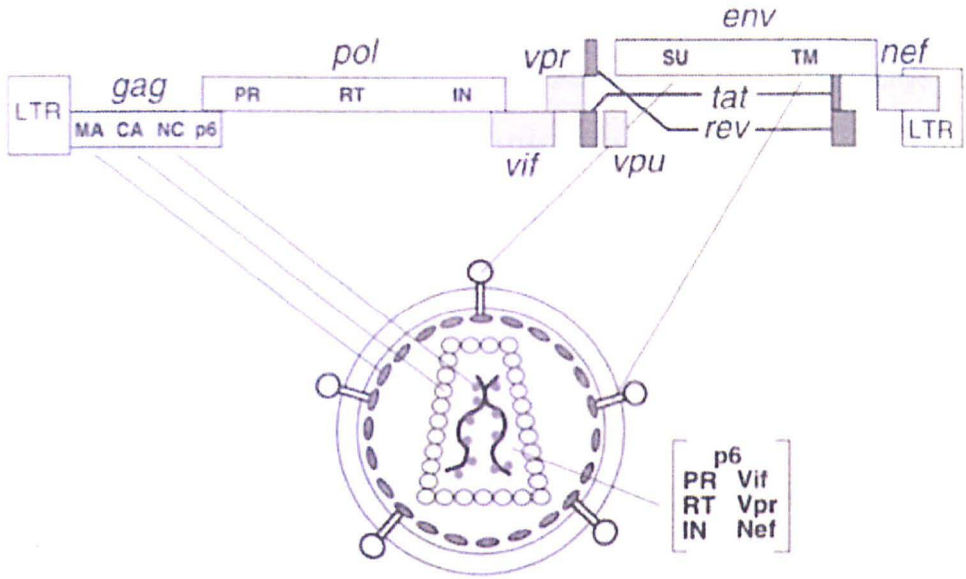

Figure 1.1: Genomic and virion structure of HIV-1 (Source: Frankel, A.D and Young, J.A.T, Annu. Rev. Biochem. 1998) [20]

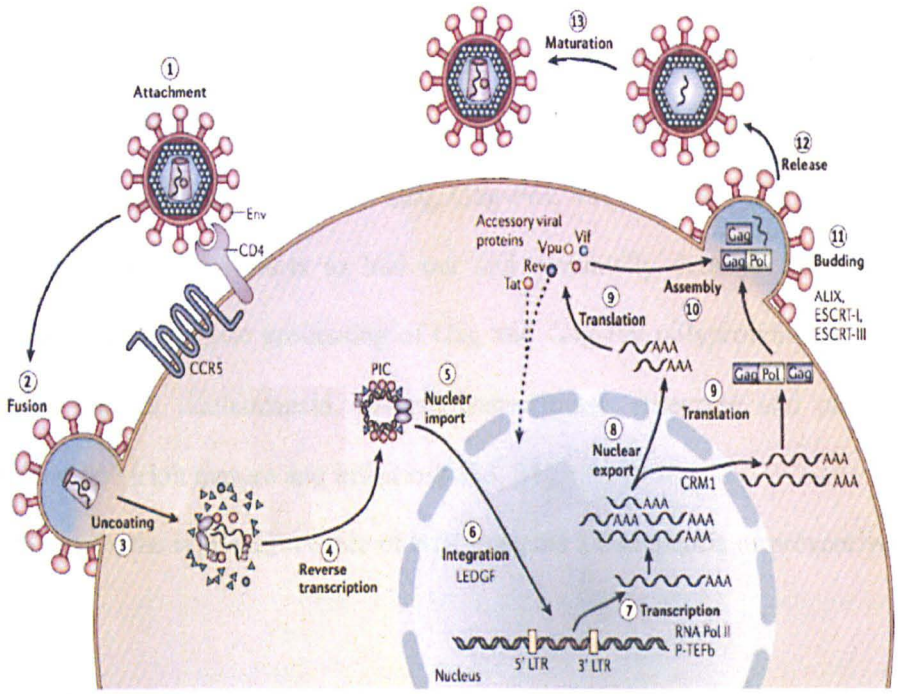

Figure 1.2: Replication cycle of HIV-1. Attachment, fusion, uncoating, reverse transcription, nuclear import, integration, transcription, nuclear export, translation, assembly, budding, release and maturation. (adapted from Engelman, A and Cherepanov, P, Nat. Rev. Microbiol. 2012)[33]

The replication cycle of HIV can be divided into 13 steps. Firstly, contact between gp120 and a CD4 molecule on the target cell leads to conformational change in the gp120, exposing the binding site for HIV-1 co-receptor molecule (CCR5 or CXCR4)(attachment). Engagement of the co-receptor molecule leads to insertion of a fusion protein and eventual fusion of the virion's envelope with the host cell's membrane, releasing the virus core into the target cell's cytoplasm where reverse transcription then starts. Successful completion of the reverse transcription at the nuclear pore triggers uncoating to allow shuttling of the pre-integration complex into the nucleus where the viral DNA is integrated into the host's genome to form a provirus. With the aid of Tat, proviral transcription is catalyzed by the host polymerase II. The transcription products are then transported from the nucleus to the cytoplasm under the regulation of Rev. Viral proteins are then translated in the cytoplasm, with Gag and Gag-Pol polyproteins localizing at the plasma membrane. Env polyprotein is translated in the endoplasmic reticulum and transported to the cell membrane. The core particle is assembled. It contains Gag, Gag-Pol, Vif, Nef, Vpr and genomic RNA. An immature virus then starts to bud out and eventually detaches from the host cell. Subsequent proteolytic processing of Gag and Gag-Pol polyproteins to give functional matrix, capsid, nucleocapsid, reverse transcriptase, integrase and protease proteins renders the virion mature and infectious [33, 34].

Each step in the replication cycle of HIV presents a therapeutic or preventive target.

1.4 Epidemiology of HIV-1

HIV is assumed to have remained unnoticed until 1981 when health professionals realized that some groups of people were suffering from an unusual condition of immune suppression. Most patients presented with Kaposi's sarcoma and *Pneumocystis jiroveci* Pneumonia (PCP), diseases that were previously associated with advanced age

or pre-existing states of immunosupression. At first, the problem was noticed among men who have sex with men (MSM) and intravenous drug users [35, 36]. Later, infants born of women in the high-risk groups [37], heterosexual contacts of the high-risk groups [38], hemophiliacs treated with blood products and recipients of multiple blood transfusion [39] were recognized as additional groups of persons that were highly likely to present with the syndrome [40]. The virus causing the syndrome was later discovered in 1983 [1, 2].

Presently, HIV is in the general population. Heterosexual sex is the primary route of transmission [41]. However, homosexual sex and intravenous drug use still remain as high-risk practices [42-44].

At the end of 2011, approximately 25 million people had died of AIDS while 34 million people, including 3.4 million children below 15 years old, were living with HIV. 69% of these people were in Sub-Saharan Africa, where 4.9% of the population was infected with HIV. Notably, the burden of the epidemic varied between regions and countries [45].

Even though the epidemic initially affected mostly men, the number of infections in women has increased considerably over time to match that of men. Currently, in Sub-Saharan Africa, approximately 61% of HIV cases are in women [46]. This high prevalence in women could be due to both socio-cultural and biological factors. For instance, access to HIV information and health services is lower in women than in men within the region, probably due to existing literacy gaps and unfavorable social norms. Furthermore, lack of economic empowerment of women in the region makes it difficult for them to negotiate for safe sex practices [45]. Structural differences between male and female genitalia could also contribute to the observed differences in infectivity. Women generally experience more exposure to infectious fluids during and after sexual intercourse due to their receptive anatomy that also presents a wider surface area. Their

higher likelihood of experiencing trauma hence breach of mucosal barrier may also contribute to a higher likelihood of infection [47, 48]. This high prevalence of HIV among Sub-Saharan African women has important implications for vertical transmission to infants. Consequently, almost 90% of all HIV-infected children worldwide live in Sub-Saharan Africa [45].

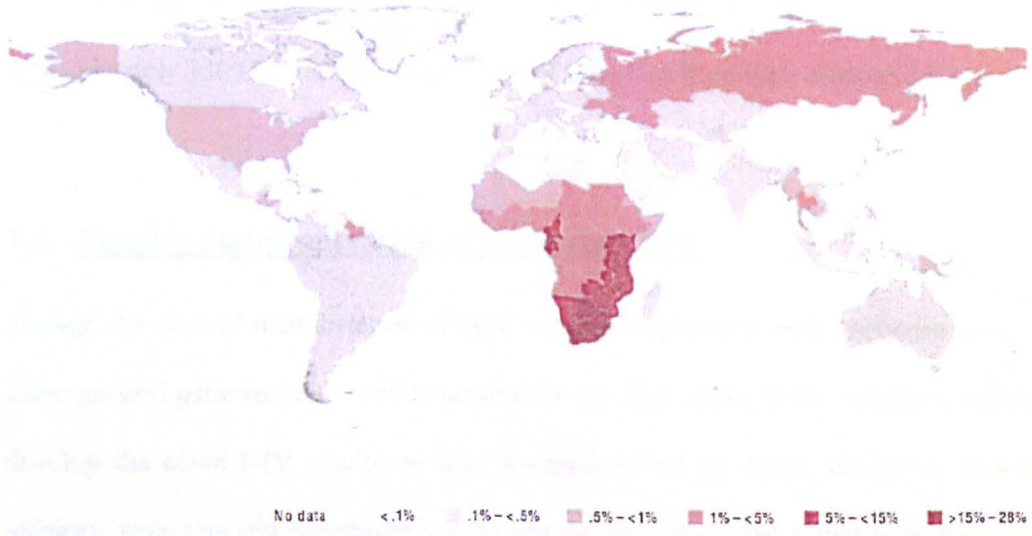

Figure 1.3: Estimated prevalence of HIV in various countries as at 2009 (Source: UNAIDS Global report 2010) [49].

Implementation of HIV preventive measures and treatment has significantly reduced both the rate of HIV-transmission and HIV-related mortality. As a result, many countries in Sub-Saharan Africa have recorded a reduction in HIV incidence [45, 50]. However, some reports suggest an increase in prevalence in some areas due to better survival of treated HIV patients after roll out of antiretroviral treatment [51]. Moreover,

the epidemic is still growing in some regions that were previously less affected e.g. North Africa and the Middle East [45].

Of significant importance is the success of prevention of mother to child transmission (PMTCT). Even though the reduction of HIV transmission in the adult population can contribute to the reduction of transmission from mothers to their children, PMTCT antiretroviral-based and infant feeding-based strategies are primarily responsible for the recent large declines in vertical transmission [52-54]. However, despite the gains in PMTCT, large numbers of children continue to be infected. For instance, in 2011 alone, approximately 330,000 children acquired HIV infection from their mothers [45].

1.5 Staging and progression of untreated HIV

Though the clinical manifestation of HIV disease progression varies between patients, some general patterns have been observed. In the first weeks of the infection, patients develop the acute HIV syndrome that is characterized by fever, headache, malaise, lethargy, myalgias and lymphadenopathy among other signs and syndromes. Very high viral loads and a sharp decline in peripheral $CD4^+$ T-cell counts characterize this period. The induction of adaptive immune responses then leads to subsequent decline in plasma viral load and partial recovery of $CD4^+$ T-cell counts [55-57]. This sets the stage for the "clinical latency" stage that can last up to a decade in some individuals. However, despite absence of clinical signs and symptoms, the "clinical latency" stage is characterized by a gradual depletion of $CD4^+$ T cells among other pathologies. In fact, viral replication continues but is maintained at a set point [58]. Eventually, due to the immune deterioration initiated in primary infection and perpetuated during the clinical latency, the patient develops end stage disease that is characterized by the onset of AIDS defining illness, appearance of opportunistic infections and a considerable increase in plasma viral load [35-40, 57, 59-61].

Due to the variability in disease progression among HIV patients, distinct patient groups have been identified. For instance, a small proportion of HIV patients maintain high $CD4^+$ T-cell counts for decades without developing end stage disease. Such individuals have been called long-term non-progressors (LTNP). Interestingly, LTNPs do not necessarily have suppressed viral loads [62-65]. On the other hand, another group of patients who control viraemia to below detectable levels in absence of treatment has been identified and termed elite controllers. Their control of viraemia is associated with transient recovery of $CD4^+$ T-cell counts and subsequent stabilization. However, not all elite controllers turn out to be LTNPs as some of them experience $CD4^+$ T-cell depletion despite viral control while others lose viral control and progress normally [64-67].

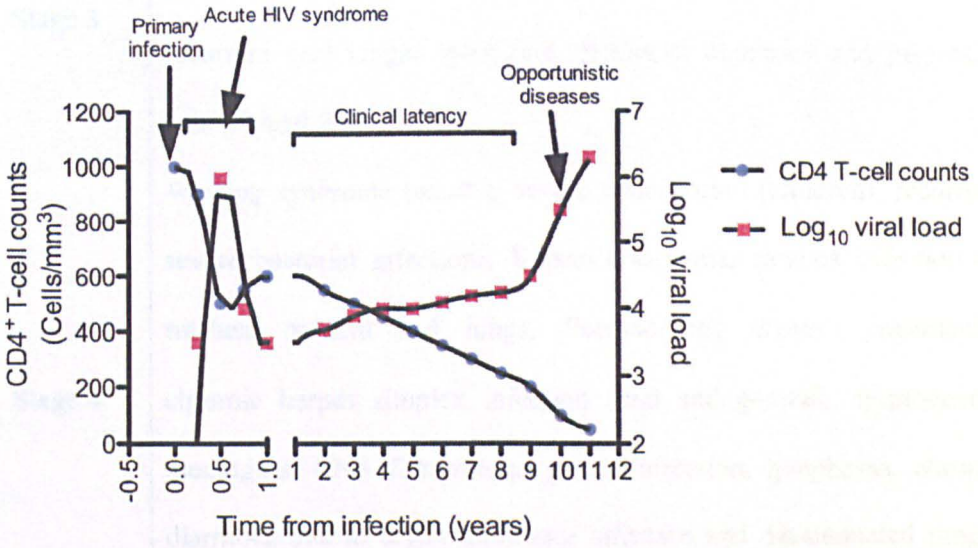

Figure 1.4: HIV disease progression in absence of treatment: Primary infection, clinical latency and end-stage disease (Adapted from Pantaleo, G. et al; NEJM 1993) [57].

Staging of HIV disease is important in determining treatment options. Currently, clinical staging and immunological staging are used for routine care. Virological staging

has not been rolled out in developing countries, primarily because of lack of the resources to implement it.

According to the WHO, HIV/AIDS clinical staging, the course of untreated HIV infection has been divided into four stages i.e. clinical stage 1 to clinical stage 4 [68].

Clinical stage	Clinical presentation
Stage 1	Asymptomatic/persistent generalized lymphadenopathy
Stage 2	Moderate weight loss, persistent hepatosplenomegaly and parotid enlargement in children, herpes zoster (shingles), skin lesions and recurrent respiratory tract infections
Stage 3	Severe weight loss (>10% of body weight) or moderate malnutrition (children), severe bacterial infections, pulmonary tuberculosis, recurrent oral fungal infections, persistent diarrhoea and persistent unexplained fever.
Stage 4	Wasting syndrome (adults), severe malnutrition (children), recurrent severe bacterial infections, Kaposi's sarcoma, candida infection of trachea, bronchi and lungs, *Pneumocystis jiroveci* pneumonia, chromic herpes simplex infection (oral and genital), cryptococcal meningitis, CNS *Toxoplasma gondii* infection, lymphoma, chronic diarrhoea due to cryptosporidium infection and disseminated fungal infections.

Table 1.1: WHO clinical staging of HIV. WHO Case definitions of HIV for surveillance and revised clinical staging and immunological classification of HIV-related disease in adults and children) [68]

WHO immunological staging of HIV infection is also divided into four levels [68].

HIV-associated immunodeficiency	Age-related $CD4^+$ T-cell values			
	<11 months (% of $CD4^+$ T cells)	**12-35 months (% of $CD4^+$ T cells)**	**36-59 months (% of $CD4^+$ T cells)**	**>5 years (Absolute CD4 numbers/mm3 or % of $CD4^+$ T cells)**
Non or not significant	>35	>30	>25	>500
Mild	30-35	25-30	20-25	350-499
Advanced	25-29	20-24	15-19	200-349
Severe	<25	<20	<15	<200 or <15%

Table 1.2: WHO immunological staging of HIV infection (adapted from: WHO Case definitions of HIV for surveillance and revised clinical staging and immunological classification of HIV-related disease in adults and children). Percentage of $CD4^+$ T cells is calculated as a proportion of total lymphocytes [68].

1.6 HIV-1 treatments

The replication cycle of HIV-1 presents several steps that can be exploited to curtail the process. A range of drugs have been developed and licensed to target some of those steps, while additional drugs are in development. The table below summarizes the details of the main licensed drugs [69].

Targeted replication step	Drug	Sub-class
Entry	Maraviroc	CCR5 binding inhibitor
	Enfuvirtide	Fusion inhibitor
Reverse transcription	Delavirdine Efavirenz Etravirine Nevirapine	Non-nucleoside reverse transcriptase inhibitors
	Abacavir Didanosine Emtricitabine Entecavir Lamivudine Stavudine Tenofovir Zalcitabine Zidovudine	Nucleoside reverse transcriptase inhibitors (NRTI)
Integration	Raltegravir	Integration inhibitor
Protease processing (virion maturation)	Atazanavir Fosamprenavir Daruavir Ritonavir Lopinavir Nelfinavir Squinavir Tipranavir Indinavir	Protease inhibitors

Table 1.3: Licenced antiretroviral drugs, their classes and HIV-replication stages to which they target [69].

These antiretroviral drugs have dramatically changed the clinical course of HIV/AIDS. Before their introduction, HIV infection was perceived as a "death sentence", but has now become a chronic infection [70]. A recent paper showed that maintaining patients at $CD4^+$ T-cell counts above 500 cells/mm^3 improves their survival considerably [71]. According to recent reports, the upscale of use of antiretroviral drugs may have also had a significant impact on the containment of the spread of the epidemic as witnessed in many countries since low viraemia correlates with a low chance of transmitting [45, 72].

Initially, antiretroviral drugs were used as monotherapies. Due to the rapid mutation rate of the virus, resistant variants quickly arose, leading to frequent treatment failures [73].

The introduction of highly active antiretroviral therapy (HAART), which comprises a combination of three drugs with different modes of action, can limit the occurrence of treatment failure and resistance. However, pre-existing drug resistance and poor compliance to the required dosing regimen can limit its usefulness. WHO recommends that the first line of treatment should combine 2 nucleoside reverse transcriptase inhibitors with 1 non-nucleoside reverse transcriptase inhibitor. Several other regimens are recommended for second line therapy [74, 75].

According to the current WHO guidelines, whose adoption varies between countries, adults are eligible for HAART if their $CD4^+$ T cell counts fall below 500 cells/μL or if they are in WHO clinical stage 3/4, whichever comes first. Children, on the other hand, become eligible for HAART if they meet any of the following criteria [76]:

1. If they are below five years of age i.e. all HIV-infected children below five years are put on HAART.
2. If they are above five years and their $CD4^+$ T-cell counts fall below 500 cells/μL or if they are in WHO clinical stage 3/4 (like adults).

Until recently, the WHO recommended all children below two years to be put on HAART the moment they were diagnosed with HIV. This is because some studies showed that untreated children maintained high viral loads and had high mortality with more than half dying before the end of two years [77-80]. However, children between two and five years were recommended to be put on HAART only if their $CD4^+$ T-cell counts fell below 750 cells/μL or below 25% or if they were in WHO clinical stage 3/4 , mainly because there was little evidence to suggest that immediate initiation of HAART conferred any biological advantages on the children who were above 2 years of age [80]. The recent revision of the guidelines to recommend immediate initiation of HAART on all children below five years was informed by the possible operational and programmatic advantages of having simplified paediatric treatment guidelines, but

evidence for any biological benefit is still lacking with regard to the two to five years age group [76].

Antiretroviral drugs are also used to prevent HIV infection. For instance, they are used to prevent transmission of the virus from infected mothers to exposed children (PMTCT)[81]. Recently, pre-exposure prophylaxis was recommended by WHO to protect people who are at high risk of HIV acquisition [82]. Antiretroviral drugs have also been long used for post-exposure prophylaxis especially on exposed health professionals and victims of sexual assault [83].

Of interest is the emerging consensus that early initiation of HAART is beneficial to the individual as well as the community. People who are started early on HAART appear to do better immunologically [84, 85]. They also transmit less, since the probability of transmitting correlates directly with viral load [86]. In fact, recent reports suggest that administration of antiretroviral drugs very early into the infection can lead to functional cure with such patients maintaining undetectable levels of the virus long after cessation of administration of the antiretroviral drugs [87, 88]. It will therefore be not surprising to see a policy change towards universal immediate initiation of HAART upon diagnosis with HIV infection. This may reduce subsequent healthcare costs at the individual level and at the same time help control the spread of the epidemic.

1.7 Immune responses and immunopathogenesis in HIV-1

Based on available evidence, most HIV-1 infections are due to a single founder/transmitted virus. Homogeneity in the early virus population also suggests that the initial stage of infection is established on a single focus of $CD4^+$ T cells in the genital mucosa [89]. Transmission is usually followed by an eclipse phase during which viraemia remains below the conventional detection limit. This phase is estimated to last about 10 days [90]. The virus eventually reaches the draining lymph nodes. Dendritic

cells have been shown to play a major role in this as they can internalize the virus via DC-SIGN in the mucosa and then transport it to the activated T cells in the lymph nodes [91]. Rapid replication of the virus ensues, with it spreading to other lymphoid organs. Of particular importance is the gut-associated lymphoid tissue (GALT), which then becomes the primary site of HIV replication. Approximately 20% of T cells in GALT are lost due to direct infection while an additional 60% die due to bystander activation and apoptosis during the acute phase of HIV infection. The numbers of $CD4^+$ T cells also fall in the peripheral circulation [92-94]. B cells in the GALT are also affected. One study showed that B cells in GALT experience apoptosis, polyclonal activation and terminal differentiation amidst loss of germinal centres and follicular damage during this acute phase. This could explain the suboptimal antibody response that is mounted against the virus particularly in the early phase of the infection [95]. This replication of the virus continues until peak viraemia is achieved 3-4 weeks after transmission [90].

The peak viraemia is associated with emergence of $CD8^+$ T cell responses. However, as the viral load decreases under the pressure of the immune responses, viral diversification occurs, leading to generation of escape mutants. The selected mutants have amino acid changes at $CD8^+$ T-cell epitopes, suggesting that the initial control of viraemia is driven by $CD8^+$ T-cell responses [96-99]. In the end, a viral set point is achieved and maintained in the chronic phase [100]. Individuals carrying HLA-B*57 and HLA-B*27 tend to have better HIV viral control, while those carrying HLA-B*35 tend to have poorer control [101, 102]. This has been suspected to be associated with the Gag-specific $CD8^+$ T-cell response among other factors. Some HLA class types could be selecting for Gag escape mutants that have reduced replicative fitness [103, 104].

The first antibody responses, in the form of immune complexes, appear approximately one week after detection of viraemia. Free antibodies against gp41 are detectable a

week later, while free antibodies against gp120 become detectable a further two weeks later. This initial antibody response is non-neutralising and appears to play no role in viral control [105]. Autologous neutralising antibodies only appear about three months or later after infection. Even though they are capable of neutralising the quasi species present at the time, rapid viral escape occurs [106, 107]. Eventually, 10-30% of HIV-infected adults develop moderate to broadly neutralising sera in the chronic phase of the infection [108]. Unfortunately, due to viral escape, they do not clear the infection. In fact, presence of broadly neutralising antibodies has been positively correlated with high viral load, suggesting that continued viral turnover could contribute to their generation [109-111]. Other suggested effector functions of antibodies in the control of HIV could include complement-mediated lyses as well as antibody-dependent cell-mediated cytotoxicity (ADCC) [112, 113].

In children, the course of HIV-infection differs considerably from that observed in adults. Most notable is the fact that high viral load is maintained throughout the first year of life in most HIV-infected children and drops only slowly with age [77, 79]. As a result, more than half of infected children die before their second birthday if left untreated [78]. The poor control of viraemia suggests that children could be having a poorer immune response to HIV infection. Indeed, early life is generally characterized by a more tolerogenic immune system. Fetal T cells, generated from a different hematopoietic stem cell lineage compared to adult T cells, tend to be more responsive to stimulants. They also have a tendency to differentiate to a regulatory T-cell phenotype [114]. Children and fetuses have higher frequencies of regulatory T cells when compared with adults, suggesting that the tolerogenic environment persists postnatally [115].

This tolerogenic situation is reinforced by the increased ability of neonatal innate immune cells to produce IL10. When exposed to Toll Like Receptor (TLR) ligands,

neonatal innate immune cells also tend to produce cytokines that support T_H2 and T_H17 differentiation that targets mainly extracellular pathogens as opposed to T_H1 responses that would target primarily intracellular pathogens like HIV [116]. However, T_H1 responses have been described in neonates who experience congenital infection with cytomegalovirus, though such responses are weaker than those observed in adults [117, 118].

Furthermore, since $CD8^+$ T-cell responses appear to play an important role in controlling viraemia, it is likely that children may miss out on the potential advantages of carrying particular HLA class 1 types that are associated with slow progression (e.g HLA-B*57 and HLA-B*27) if the transmitted virus has already been adapted to those HLA alleles in the mother [101, 102, 119]. Also, the sustained high viral load may lead to accumulation of compensatory mutations that can negate any loss of fitness that would result from $CD8^+$ T-cell-mediated Gag mutations [120].

1.8 Systemic immune activation in HIV

Systemic immune activation is one of the most important phenomena associated with HIV infection. It has been described in almost all compartments of the immune system in untreated HIV patients. In the T-cell compartment, both CD4+ and CD8+ T cells from HIV patients have increased expression of markers of activation, namely CD38 and HLA-DR [121]. Hypergammaglobulinaemia, the production of large amounts of immunoglobulins that are not targeted to any particular antigen, is the classical indicator of aberrant polyclonal activation of B cells in HIV [122]. B cells from HIV patients have also been shown to express increased levels of markers of activation and terminal differentiation [123]. The innate compartment is not spared either, as HIV has been shown to directly activate plasmacytoid dendritic cells (pDCs), leading to production of large amounts of cytokines that could modulate the adaptive arm [124].

The consequence of immune activation is a constant supply of activated T cells that are targets for infection [125]. Secondly, the uncontrolled activation is suspected to be an immune escape strategy that the virus employs to blunt the immune response against itself by causing exhaustion and apoptotic depletion of various lymphocyte subsets [126, 127]. Interestingly, $CD4^+$ T-cell depletion has been observed in elite controllers and shown to correlate with their levels of T-cell activation despite their undetectable viral loads [128]. Furthermore, HAART treated patients who maintain high levels T-cell activation despite control of viraemia have poorer recovery of $CD4^+$ T-cell counts [129]. More importantly, the level of T-cell activation is a reliable predictor of progression to AIDS [130]. Unlike non-pathogenic SIV, pathogenic SIV models have shown similar patterns of immune activation when compared with HIV in humans, further underscoring the importance of immune activation in pathogenesis [131].

Despite intensive research, the causes of the immune activation in HIV have not been fully elucidated. However, it has been shown that viral factors could play, at least in part, an important role. The HIV virion has several known activators of immune cells. The envelope glycoprotein has been shown to have activatory effects on B cells and monocytes via DC-SIGN [132]. The viral genome, ssRNA, has also been shown to activate pDCs via toll like receptors 7 [124]. HIV Nef, one of the accessory proteins, has been shown to activate monocytes and consequently indirectly activate B cells via ferritin from the activated monocytes [133]. All these activatory effects lead to production of soluble and contact factors that could cause the activation observed in the T and B-cell compartments.

The observation of some continued immune activation in elite controllers and HAART treated patients without detectable viraemia suggest that the observed activation could also be due to non-viral factors [128, 129]. This argument is further supported by the fact that non-pathogenic SIV models show low levels of chronic immune activation when compared with pathogenic SIV models despite both presenting with comparably high viral loads, implying that the activation in pathogenic models could be independent from direct viral replication [131]. Alternatively, as has been argued in one study, the non-pathogenic SIV models could be missing some pathways that are activated by the HIV factors [134].

Microbial translocation from the gut has been proposed as the mechanism by which HIV mediates systemic immune activation independently from HIV factors. Systemic levels of LPS have been shown to correlate with systemic immune activation [135]. Even though LPS only activates cells in the innate immune system, the subsequent release of inflammatory factors could have activating effects on cells in the adaptive arm of the immune system. Furthermore, the presence of LPS in circulation is an indication of the possible translocation of other bacterial components that could have a direct effect on T and B cells, e.g. bacterial DNA could activate B cells via toll like receptor 9.

Despite the technical challenges in accessing appropriate biological samples from the gut, a few studies have successfully revealed valuable information on the effect of HIV infection on the gut. HIV has been shown to significantly compromise both the structural and the immunological barrier in the gut. For instance, severe depletion and dysregulation of T and B cells in the gut associated lymphoid tissue (GALT) has been shown to start in the acute phase of the infection and is maintained in the chronic phase [93, 95]. In addition, increased intestinal permeability, impaired digestion and impaired

absorption characterise the accompanying enteropathy [136-138]. The increased permeability, together with the destruction of the immunological defences in the gut, would arguably allow the resultant microbial translocation.

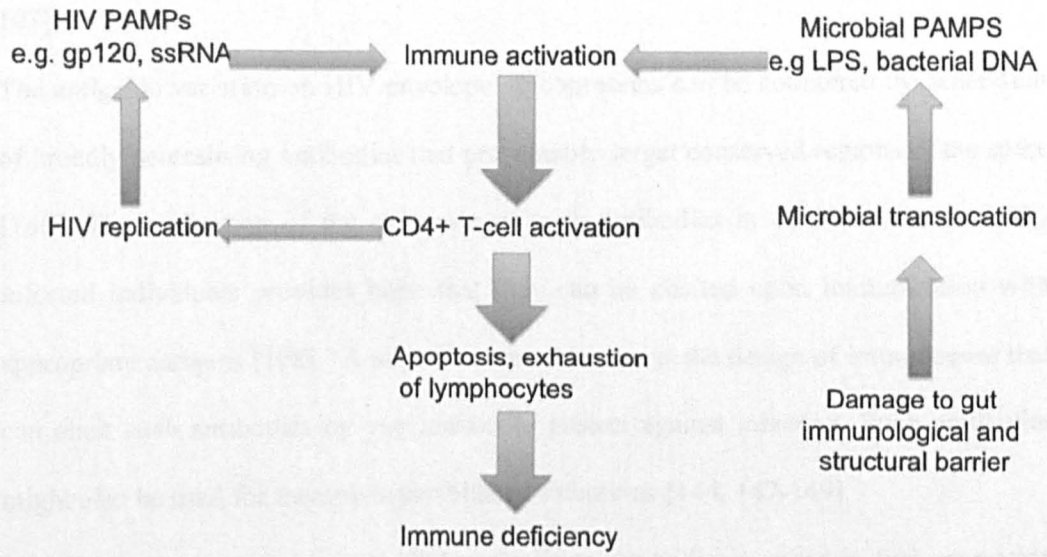

Figure 1.5: Summary of the events that could account for the systemic immune activation that is observed in HIV. PAMPs-pathogen associated molecular patterns [139].

1.9 Broadly neutralising antibodies

Currently, the generation of neutralising antibodies is of primary research interest. . Such antibodies targeting the HIV spikes (envelope glycoproteins) have been shown to effectively control viraemia as well as provide protection from infection by HIV and SIV in animal models [140-144]. Furthermore, administration of broadly neutralising antibodies to humans has been shown to delay viral rebound upon interruption of antiretroviral therapy [144].

However, HIV has several immune evasion strategies, the most notable being the presence of an error-prone reverse transcriptase that gives rise to a high degree of antigenic variation both within the host and at the population level. As a result, there is

up to 35% variation between different HIV-1 clades and up to 20% variation between viruses within the same clade [145]. Consequently, the neutralising antibodies that are produced, albeit late (several months after infection), tend to neutralise only the autologous virus that in turn rapidly mutates to neutralisation-resistant phenotypes [106, 107].

The antigenic variation on HIV envelope glycoproteins can be countered by generation of broadly neutralising antibodies that presumably target conserved regions of the spike [146]. The realization of the existence of such antibodies in 10-30% of chronically infected individuals provides hope that they can be elicited upon immunization with appropriate antigens [108]. A major current objective is the design of immunogens that can elicit such antibodies by vaccination to protect against infection. Such antibodies might also be used for therapy in established infections [144, 147-149].

Initially, attempts to isolate anti-HIV neutralising antibodies resulted in first generation broadly neutralising antibodies that were, to some extent, limited in either breadth or potency. However, recently, through a renewed international effort, a number of second-generation broadly neutralising antibodies have been isolated from HIV-infected patients whose sera show broad neutralising activity. The table below summarizes the details of some of the broadly neutralising antibodies that have been isolated to date.

Antibody	Specificity	Breadth at IC_{50}<50 µg/ml	Year	Reference
b12	gp120CD4bs	34%	1991	[150, 151]
2F5	gp41MPER	57%	1994	[152, 153]
4E10	gp41MPER	96%	1994	[151, 152]
2G12		32%	1994	[151, 152]
PG9	Glycans & V1/V2	78%	2009	[153, 154]
PG16	Glycans & V1/V2	73%	2009	[153, 154]
HJ16	gp120CD4bs	40%	2010	[155]
VCR01	gp120CD4bs	91%	2010	[156]
VRC02	gp120CD4bs	91%	2010	[156]
VRC03	gp120CD4bs	50%	2010	[156]
PGT121	Complex type V3 N-glycans	70%	2011	[151, 157]
PGT122		65%	2011	[151]
PGT123		67%	2011	[151]
PGT126		60%	2011	[151]
PGT128	Glycans and V3 beta strand	72%	2011	[151]
PGT145	Glycans and V1/V2	78%	2011	[151]
CH01-	Glycans and V1/V2	50%	2011	[158]
3BNC117	gp120CD4bs	84%	2011	[153, 159]
NIH45-46	gp120CD4bs	85%	2011	[153, 159]
VRC-CH30	gp120CD4bs	83%	2011	[160]
VRC-PG04	gp120CD4bs	76%	2011	[160]
10E8	gp41MPER	98%	2012	[153]
3BC176	CD4i/V3	60-70%	2012	[161]
J3	gp120CD4bs	96%	2012	[162]

Table 1.4: Details of some in vitro broadly neutralising antibodies isolated to date. Their specificities, breadths and years of publication are shown. The publications reporting their isolation are referenced.

These broadly neutralising antibodies are currently being used to inform structure-based vaccine design. For instance, introduction of alanine and glycan residues has been done to direct immune responses to b12 binding sites of gp120 [163-165]. Also, identification of the b12 binding epitopes on the gp120 outer domain (OD) has enabled the design of OD constructs in a bid to generate better responses against the b12 binding epitopes that are poorly immunogenic in the context of intact gp120 [166]. In addition, the mapping of the binding site of 2G12 on the glycan shield has informed the development of glycoconjugates with the aim of eliciting 2G12-like antibodies [167]. The target epitopes for 2F5 and 4E10 on gp41MPER have also been mapped and used to design

peptides that are meant to elicit anti-gp41 broadly neutralising antibodies [168]. However, thus far, all these strategies have yielded disappointing results. Further studies on the possible mechanism through which some HIV patients generate broadly neutralising antibodies are expected to inform novel and more successful strategies.

1.10 HIV vaccine development

HIV vaccine development has proven to be a daunting task. The biggest obstacles are the high degree of antigenic variation of the virus that renders vaccines unprotective against field isolates [145, 169]; and the absence of an effective immune response capable of completely controlling the natural infection, with the exception of few elite controllers and long-term non-progressors, has made it difficult to identify definitive correlates of protection and viral control [170].

Most of the HIV vaccine development effort in the past decade has focused on eliciting anti-HIV $CD8^+$ T-cell responses. This is partly because previous antibody based vaccines failed to elicit protective neutralising antibodies [171, 172]. In addition, $CD8^+$ T-cell responses have been shown to play a crucial role in the partial control of viraemia during acute HIV infection and in chronic infection in patients carrying some HLA 1 alleles [96, 97, 99, 101, 102]. However, the failure of a purely T-cell based vaccine in the STEP trial coupled with the realization that a subset of chronically HIV-infected individuals naturally develop broadly neutralising antibodies has rekindled the interest in developing antibody based vaccines [108, 173]. Moreover, RV144, the only HIV vaccine to show some efficacy, did not elicit any $CD8^+$ T cell responses but had detectable $CD4^+$ T cell responses and antibody responses [174], suggesting that a combination of responses may confer better efficacy.

$CD8^+$ T cells respond to antigen-derived peptides that are presented on MHC class I molecules. This is best accomplished if the antigen of interest is produced endogenously, even though exogenously produced antigens can also be presented via MHC class I, but less efficiently, by cross-presentation [175]. To achieve endogenous production of HIV antigens in antigen presenting cells, viral vectors and plasmid DNA have been used to deliver the genetic codes of antigens into host's cells [173].

The administration of plasmid DNA can be manipulated to elicit the desired $CD4^+$ T cell responses that may act as appropriate helper cells to either $CD8^+$ T cells or B cells in subsequent immunizations [176]. As a result, DNA vaccines have mainly been used to prime the immune system in prime-boost regimens. Unfortunately, they have poor immunogenicity when compared to other platforms. However, in vivo electroporation as well as incorporation of molecular adjuvants (cytokine and chemokines) have been shown to enhance their immunogenicity [177, 178]. A considerable advantage of using plasmid DNA has been the lack of vector-directed immune responses in vaccinees.

The most widely used viral vectors are poxvirus and adenovirus based vectors. The most commonly used poxvirus vectors are modified *Vaccinia ankara* (MVA) and ALVAC. Poxvirus vectors tend to express other vector proteins that may focus the immune response away from the HIV antigen of interest, whereas adenovirus vectors can be easily engineered to express only the HIV antigen of interest [179]. However, preexisting immunity against the various serotypes of adenovirus may greatly influence their immunogenicity [180]. To address this problem, chimpanzee adenoviruses that humans rarely encounter naturally are now being used with relatively good success [181, 182].

Previous protein-based vaccines that failed to generate broadly neutralising antibodies consisted of recombinant envelope glycoproteins expressed from particular laboratory and field strains [171, 172]. Following the recent intensive investigation of B-cell

biology and properties of HIV-1 glycoproteins, coupled with the isolation of several broadly neutralising antibodies that inform on the most promising targets for neutralisation, the design of HIV protein immunogens has taken a rational form [183]. Immunogens have been designed to focus the immune response to neutralisation sensitive epitopes as well as to limit the flexibility of the glycoprotein among other strategies [184].

To date, three phase III efficacy HIV-1 vaccine trials have been reported. The first trial, named VAX004, evaluated AIDSVAX B/B that contained two recombinant gp120 antigens, one from the MN laboratory strain and the other from the GNE8 primary isolate. The vaccine did not show any protective efficacy, but peak antibody response among vaccinees seemed to negatively correlate with HIV-1 incidence [171, 185]. The second phase III efficacy trial, VAX003, was done in Thailand among intravenous drug users to evaluate AIDSVAX B/E that contained two recombinant gp120 antigens, one from the CXCR4-dependent MN laboratory clade B strain and the other from a CCR5-dependent CRF01_AE primary isolate. It, too, did not show significant efficacy [172]. The third phase III efficacy trial, RV144, evaluated a prime-boost regimen whereby vaccinees were primed with a series of doses of ALVAC-HIV (vCP1521) followed by boosting with AIDSVAX B/E. ALVAC-HIV (vCP1521) is a recombinant Canarypox vectored vaccine that encodes for gag, pro and gp120 linked to the transmembrane anchoring portion of gp41. The RV144 trial showed a protective efficacy of 31.2%, proving for the first time that HIV-1 infection can be prevented by vaccination in humans [174]. Subsequent analysis for immune-correlates revealed that binding of IgG to V1/V2 region of gp120 correlated inversely with rate of infection while binding of plasma IgA correlated directly with rate of infection [186].

Many more HIV vaccines have been tested in preclinical, phase I and phase II trials with varying outcomes. Notable among them is MRKAd5 HIV-1 gag/pol/nef vaccine

that was evaluated in the Phase IIb STEP study on individuals at high risk of HIV acquisition. It comprised of 1:1:1 mixture of three replication-defective Ad5 vectors, one each encoding for gag, pol and nef. Interim analysis showed that the vaccine neither protected from infection nor controlled viral load upon infection, prompting termination of the study. Surprisingly, there was an increase in the rate of HIV infection among vaccine recipients who were uncircumcised or had pre-existing high titres of anti-vector antibodies [173]. A similar phase IIb trial that comprised of Ad5 vectors encoding for Gag, Pol, Nef and Env proteins, HVTN 505, also showed lack of efficacy despite the fact that individuals who had high titres of anti-vector antibodies were excluded in the recruitment [187].

1.11 B-cell biology

B cells are the lymphocytes that are responsible for the production of antibodies. They also produce cytokines that affect other immune cells during immune responses.

Several receptors that play a role in the activation of human B cells have been described. They include B cell receptor (BCR), CD40, Toll like receptors (TLRs) and cytokine receptors among others.

The BCR is a transmembrane receptor that interacts with the cognate antigen for the particular B-cell clone and initiates the B-cell response. It consists of two moieties namely [188]:

(a) Surface immunoglobulin that acts as the ligand binding moiety

(b) Signal transduction moiety consisting of two components, Igα and Igβ.

CD40, on the other hand, is a membrane bound receptor belonging to the TNF-receptor superfamily. It is expressed on B cells, dendritic cells and macrophages among other cells. Its natural ligand is CD40 ligand (CD40L) that belongs to the TNF-superfamily

and is expressed on the surface of activated helper $CD4^+$ T cells [189-191]. The CD40-CD40L interactions between T cells and B cells are crucial in the generation of optimal antibody responses as evidenced in humans that have defective CD40 signalling and subsequently have defective antibody responses [192].

Another important signal in activation of B cells is mediated via Toll like receptors (TLRs), germ line encoded receptors of the innate immune system that were so named due to their similarity with the toll protein in drosophila. They recognise conserved structural patterns on pathogens (pathogen associated molecular patterns-PAMPs) and like the toll protein, they initiate innate immune responses against the invading pathogens. They belong to a class of receptors called pattern recognition receptors (PRRs) [193-196]. Ten different toll like receptors have been identified in humans, TLR1-TLR10. They are mainly found in the cells of the innate immune system, though some are also expressed in B cells [197, 198]. In addition to initiating innate responses, they have been shown to significantly modulate the adaptive immune response [199-201]. Human B cells express TLR1, TLR2, TLR6, TLR7, TLR9 and TLR10 [197, 198, 202]. Of particular interest for this thesis are TLR7 and TLR9 that are located in endosomal compartments and act as ligands for single stranded RNA and DNA, respectively [203-205]. They are located intracellularly in endosomal compartments so that they can detect the genetic material of pathogens after degradation of the pathogens by intracellular lytic enzymes. Their ligation by pathogen DNA or RNA initiates signalling via NFκB that eventually influences the expression of various proinflammatory genes, some of which play a role in influencing the antibody response [206].

Cytokine receptors have been shown to strongly modulate the effect of CD40L, TLR ligands and BCR ligands on B cells. For instance, IL21 is the most potent cytokine that is produced by activated T cells to influence the proliferation and differentiation of B

cells during an immune response [206]. It also promotes the antitumor effect of natural killer cells and cytotoxic T cells [207, 208]. It is a member of IL-2 family of cytokines, which also comprises IL-2, IL-4, IL-7, IL-9 and IL-15. Its receptor, IL-21R, is generally expressed on B cells, but with varied expression levels in different B-cell subsets [209, 210].

In an ideal T-dependent antibody response, the activation of B cells starts with the interaction of the B cell receptor (BCR) with the cognate antigen on the invading pathogen. The binding of the antigen onto the BCR leads to two outcomes. Firstly, the BCR delivers signals via various intracellular signalling pathways. Secondly, the BCR delivers the antigen to intracellular endosomal compartments by a process of receptor-mediated endocytosis. In some instances where the antigen is part of a whole pathogenic organism, the process may lead to internalization of the whole pathogen [206]. The antigen is then degraded into peptides, among other components, leading to release of any associated genomic material that can act as ligand to the endosomally located toll like receptor (TLR) 7 and TLR 9 that recognise single stranded viral RNA and bacterial DNA, respectively. Signalling via toll like receptors therefore leads to a second signal in the responding B cell [211]. The peptides that are generated from the degradation of the antigen are then loaded onto MHC-II molecules and displayed on the surface of the B cell. The peptide:MHC-II complexes are recognised by the T cell receptors (TCR) of cognate helper T cells that have been primed by dendritic cells that display the same peptides after encountering the same antigen, a phenomenon called linked recognition. This interaction leads to the provision of T-cell help onto the B cell via contact and soluble factors. Most notable is the interaction of membrane-bound CD40 ligand on the T cells with CD40 on the surface of the B cell. CD40-CD40L interaction has been shown to be a crucial additional (third) signal that dictates the fate of the responding B cell [206]. Cytokines (e.g. IL21 and IL4) produced by the helper T

cells also provide an additional (fourth) signal that modulates the response of the B cells [212].

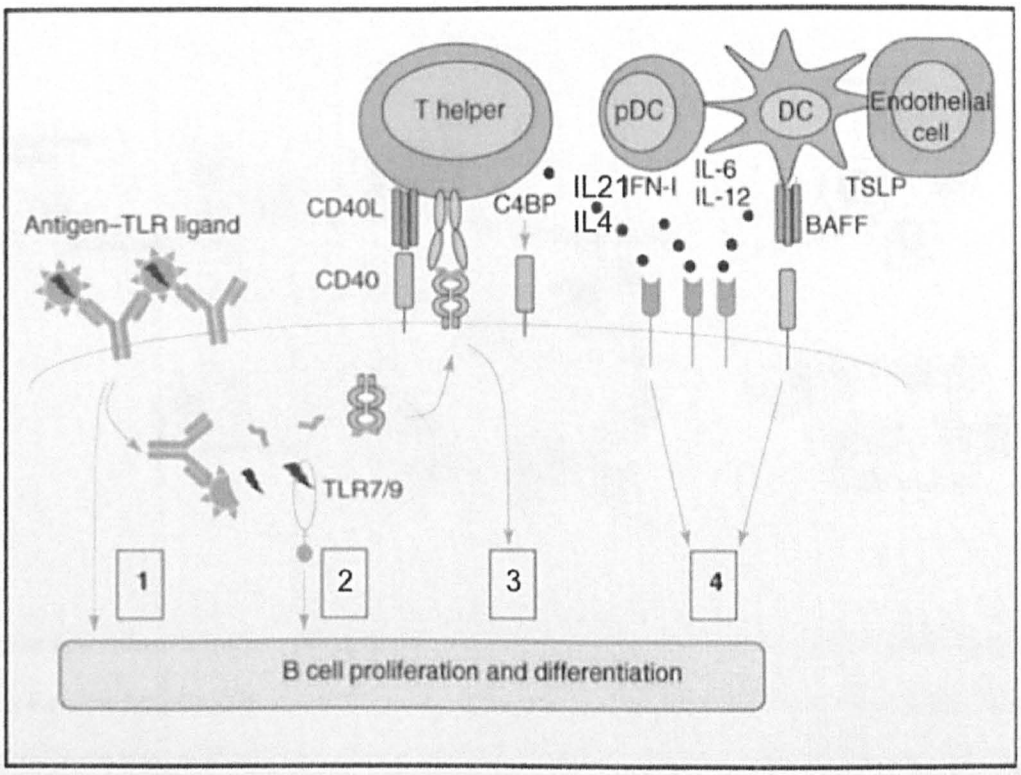

Figure 1.6: Activatory signals that influence B cells during an immune response. Signal 1-BCR cross linking; Signal 2-signalling via toll like receptors; Signal 3-contact T-cell help; Signal 4-signalling via cytokine from helper T cells or innate cells (Adapted from Lanzavecchia et al [211]).

When B cells are activated via the above-described signals, the quality and magnitude of the response is greatly influenced by the processes that take place in germinal centre microenvironments. Germinal centres are anatomical sites in lymphoid organs where B cell-T cell interactions occur. Normally, when a B cell encounters its cognate antigen, it undergoes clonal expansion. Some of the daughter cells may differentiate extra-follicularly into short-lived plasma cells that secrete mainly IgM. Others may participate in a germinal centre reaction where they undergo several rounds of somatic hyper-mutation of their immunoglobulin genes and Darwinian selection, leading to antibody avidity maturation. Concurrently, they also undergo class-switch recombination, leading

to expression of switched immunoglobulin isotypes. Eventually, the cells differentiate into either long-lived plasma cells or memory B cells (Figure 4.1) [213].

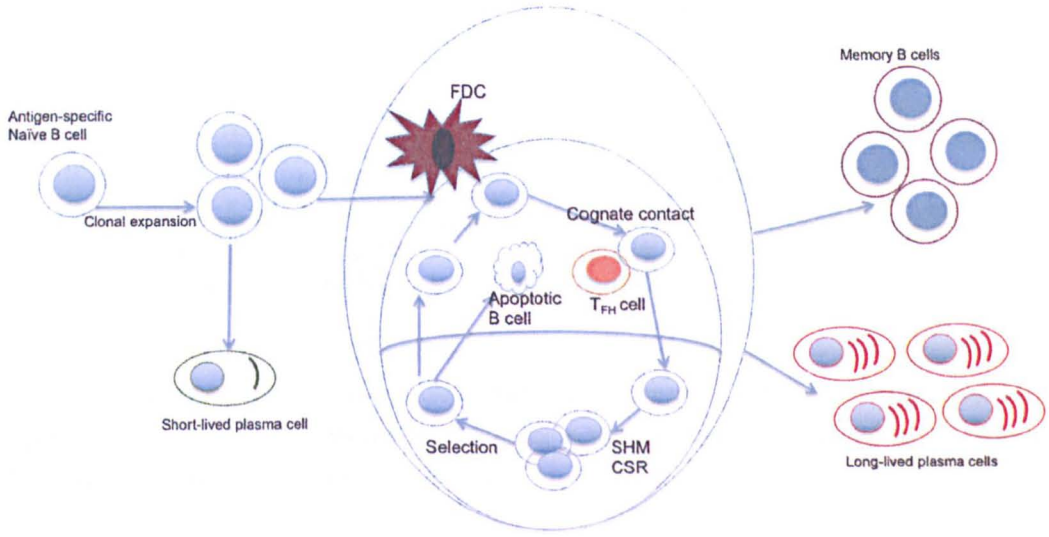

Figure 1.7: An ideal B-cell response. B cells go through clonal expansion, extra follicular differentiation into short-lived plasma cells, germinal centre reaction consisting of somatic hyper mutation (SHM), class switch recombination (CSR), and selection with the eventual production of long-lived plasma cells and memory B cells [213].

Thus, the germinal centre reaction influences the quality of antibody response by enabling avidity maturation and class switching from IgM and IgD expression to IgG, IgE and IgA expression. Depending on the cytokine environment, the IgG class switching could be to any of the subclasses IgG1, IgG2, IgG3 or IgG4 [206].

Role of cytokines in regulating expression of antibody classes							
Cytokines	**IgM**	**IgG3**	**IgG1**	**IgG2b**	**IgG2a**	**IgE**	**IgA**
IL-4	Inhibits	Inhibits	Induces		Inhibits	Induces	
IL-5							Augments production
IFN-γ	Inhibits	Induces	Inhibits		Induces	Inhibits	
TGF-β	Inhibits	Inhibits		Induces			Induces

Figure 1.8: Effect of various cytokines on the secretion of different immunoglobulin isotypes and sub-classes by mouse B cells. Red-inhibits, violet-induces, blue-augments.The inhibitory effect on secretion of particular isotypes or sub-classes is as a result of the mutually exclusive induction of other isotypes or sub-classes (From Janeways Immunobiology [206]).

Avidity maturation leads to production of antibodies that bind their target antigen more avidly and therefore have better efficacy in controlling the infection. On the other hand, class switching confers new effector functions that are critical in the efficacy of the antibodies to control the target infections as summarised in the table below [206].

Functional activity	**IgM**	**IgD**	**IgG1**	**IgG2**	**IgG3**	**IgG4**	**IgA**	**IgE**
Neutralization	+	-	++	++	++	++	++	-
Opsonization	+	-	+++	*	++	+	+	-
Sensitization for killing by NK cells	-	-	++	-	++	-	-	-
Sensitization of mast cells	-	-	+	-	+	-	-	+++
Activates complement system	+++	-	++	+	+++	-	+	-

Figure 1.9: The effector functions of various human immunoglobulin isotypes and subclasses. +++/Red-major effector function, ++/dark pink-lesser effector function, +/light pink-minor effector function. * IgG2 opsonisation effect is dependent on presence of a certain Fc receptor allotype (From Janeways Immunobiology [206]).

In some pathological conditions like HIV and malaria, other than naïve and classical memory B cells, other B-cell subsets have been detected in significant frequencies in peripheral circulation. Some of these subsets have been previously described in the developmental pathway of B cells e.g. HIV infection in adults has been associated with increased frequencies of immature/transitional B cells, plasmablasts and activated mature B cells. However, a newly described B-cell subset has also been detected in HIV and malaria patients as opposed to healthy controls. These cells have been referred to as atypical memory B cells or tissue-like memory B cells or exhausted memory B cells [214, 215].

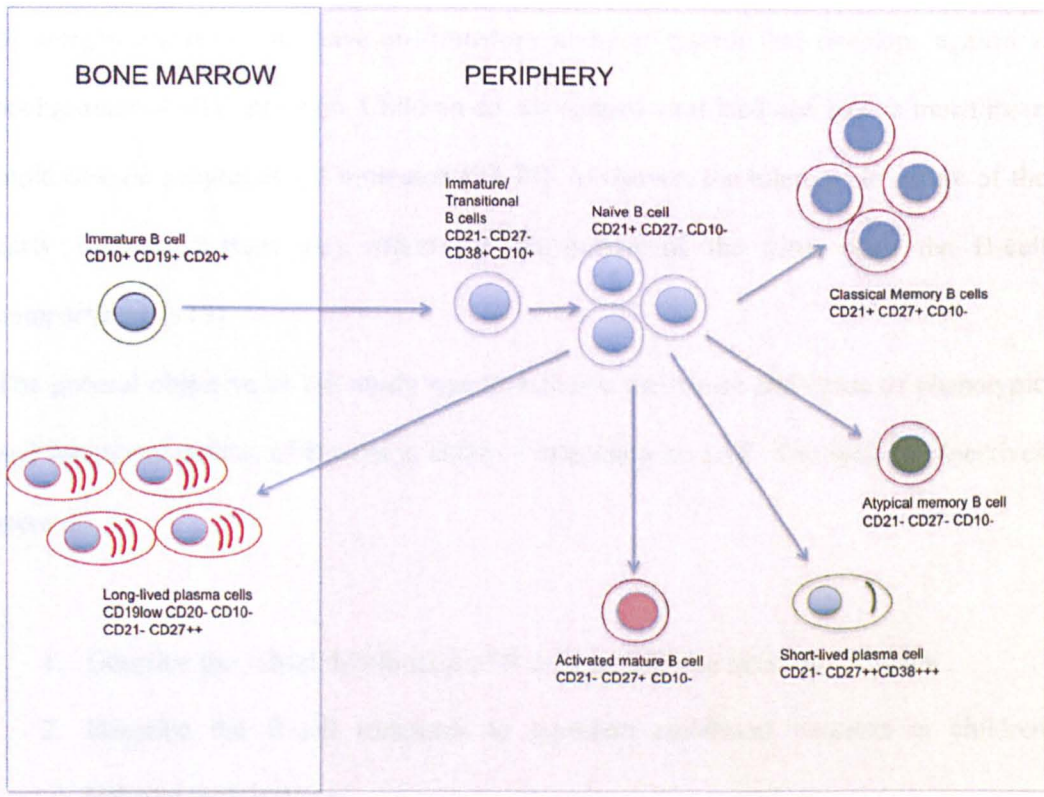

Figure 1.10: Summary of the possible differentiation pathways that B cells may go through both in health and disease [214, 215].

1.12 Justification for the study

With the renewed interest in developing antibody-based HIV vaccines, it is important to understand the interactions of the virus with the B-cell compartment. Current literature suggests that HIV has deleterious effects on B cells, either directly or indirectly, and it could benefit from some of these effects which blunt antibody responses against itself [215].

Detailed descriptions of the B-cell defects in adults infected with HIV have been reported [84, 215]. However, descriptions of B cell defects in children have not been done to the same depth. Unlike adults who become infected in adulthood when their immune systems have already matured, children differ in that they have less experience of antigen exposure and have an immature immune system that develops against a background of HIV infection. Children do not control viral load and have a much more rapid disease progression if untreated [77-79]. Moreover, the tolerogenic nature of the early immune system may affect the interaction of the virus with the B-cell compartment [115].

The general objective of this study was to describe the nature and cause of phenotypic and functional defects of B cells in children infected with HIV. The specific objectives were to:

1. Describe the subset distribution of B cells in children infected with HIV.
2. Describe the B-cell responses to common childhood vaccines in children infected with HIV i.e.
    a. Quantities of antibodies in circulation.
    b. Avidities of antibodies in circulation.
    c. Frequencies of memory B cells in peripheral blood.

3. Describe the memory B-cell responses to pneumococcal capsular polysaccharides in children after natural exposure or vaccination with a pneumococcal conjugate vaccine.
4. Determine the in vitro response of B cells to different B-cell stimulant-combinations and evaluate the in vitro effects of recombinant HIV-1 Nef protein on B-cell responses to such stimulant-combinations.

Chapter 2 Materials and methods

2.1 Study site

HIV-infected children were recruited from the Comprehensive Care and Research Clinic (CCRC) in Kilifi District Hospital (KDH). The hospital is located in Kilifi along Kenya's Indian Ocean coast, approximately 60KM north of Mombasa. Community controls were recruited from the 'Ngerenya cohort', a low endemicity malaria epidemiological surveillance cohort located to the north of KDH [216]. Additional children were recruited from the Pneumococcal non-typeable *Haemophilus Influenza* protein D conjugate vaccine (PHiD-CV) Reactogenicity and Immunogenicity Study in nearby Malindi (PRISM) to evaluate the acquisition of B-cell memory after natural exposure to pneumococcal antigens and after adminstration of the pneumococcal conjugate vaccine [217]. Biological samples from all study participants were analysed at the KEMRI-Wellcome Trust Research Programme laboratories.

In vitro experiments to evaluate the effect of different stimulants on B cells and effects of pre-incubation with recombinant HIV-1 Nef on B-cell responses to the stimulants were done in Dr Britta Urban's laboratory at Liverpool School of Tropical Medicine. Buffy coats obtained from healthy blood donors were purchased from the National Health Service Blood and Transplant service.

2.2 Ethical considerations

The study was approved by the Kenya Medical Research Institute (KEMRI) Ethical Review Committee (ERC) under protocols SSC No: 1131, SSC No: 1633 and SSC No: 1635. Written informed consent was obtained from the parent/guardian of every participating child.

Buffy coats were ordered from National Health Service Blood and Transplant service under the Liverpool School of Tropical Medicine Research Tissue Bank Approval

(REC ref 11/H1002/9) and were held under the Human Tissue Authority license number 12548.

2.3 Study designs

The CCRC study was a cross-sectional study aimed at recruiting children of comparable ages belonging to the four treatment groups as shown in figure 2.1.

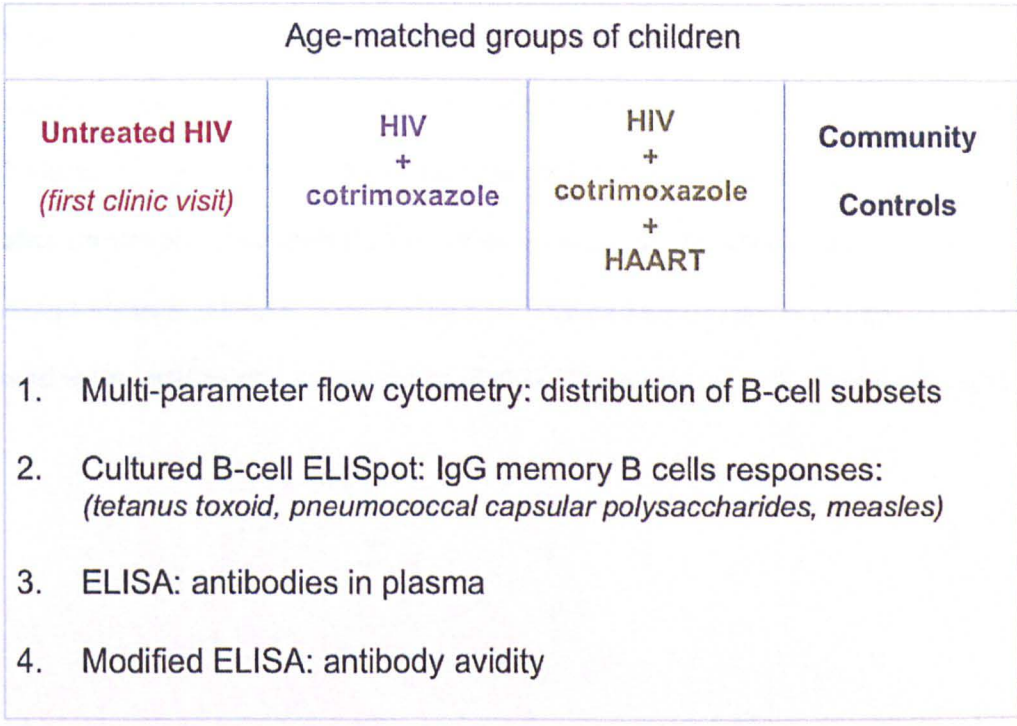

Figure 2.1: Cross-sectional design of the study comparing HIV-infected children at CCRC with community control children in Ngerenya

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The PRISM study was a double blind randomized controlled clinical trial. It evaluated immunogenicity and reactogenicity to a PHiD-CV vaccine in children aged 12-59 months. In a memory B cell sub-study, PBMCs from 35 participants in group B (see Table 2.1) aged 12 to 24 months were collected on the day of enrollment (just before the first dose), 30 days after enrollment (1 month after first dose) and 210 days after enrollment (1 month after second dose) and were used to evaluate the frequencies of memory B cells against tetanus toxoid, diphtheria toxoid and some of the pneumococcal capsular polysaccharides in the PHiD-CV vaccine. Therefore, the sub-study was a longitudinal study whereby participants were followed up after receiving the PHiD-CV vaccine on the day of enrollment, DTaP (Diphtheria and Tetanus toxoids and acellular Pertusis) vaccine 60 days after enrollment and a second dose of PHiD-CV 180 days after enrollment. Data generated by other scientists in the randomized clinical trial on nasopharyngeal pneumococcal carriage and plasma anti-pneumococcal IgG levels were used in the analysis and interpretation of the ELISpot data generated in the sub-study.

	Group A N=200	Group B N=200	Group C N=200
Day 0	PHiD-CV	PHiD-CV	Hepatitis A vaccine (Havrix)
Day 60	PHiD-CV	DTaP (Infanrix)	DTaP (Infanrix)
Day 180	DTaP (Infanrix)	PHiD-CV	Hepatitis A vaccine (Havrix) and PHiD-CV

Table 2.1: The vaccination schedule in the PRISM study: DTaP-Diphtheria and Tetanus toxoids and acellular Pertusis, PHiD-CV- Pneumococcal non-typeable *Haemophilus Influenza* protein D conjugate vaccine.

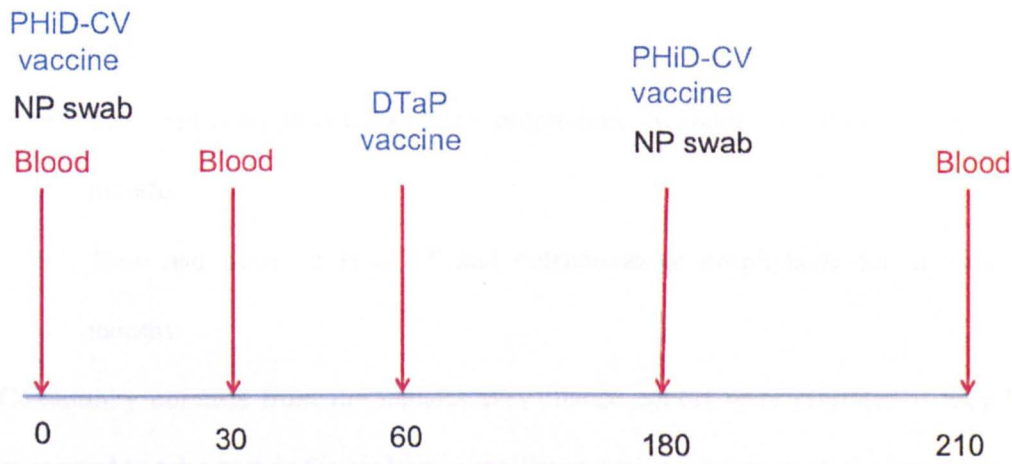

Figure 2.2: Longitudinal design of the memory B cell sub-study showing time points when participants received the PHiD-CV, received a nasopharyngeal swab or donated a blood sample. Cultured B-cell ELISpots were done on the PBMCs that were isolated from the blood samples.

In the in vitro assays, PBMCs were isolated from buffy coats to determine the effects of various stimulant-combinations on B cells. The effects of recombinant HIV-1 proteins were assessed by pre-incubating the PBMCs with the recombinant proteins and then stimulating them with the various B-cell stimulant-combinations.

2.4 Criteria for inclusion of study participants

HIV-infected children 18 months to ten years who were registered in the CCRC were recruited if they consented and met any of the following criteria.

- They had not started cotrimoxazole prophylaxis i.e. on their first visit to the CCRC.
- They had been on cotrimoxazole prophylaxis (without HAART) for at least six months.
- They had been on HAART and cotrimoxazole prophylaxis for at least six months.

Community controls from the malaria surveillance cohort were recruited if they had consented to take part in the malaria surveillance and if they were of similar age to the HIV-infected children recruited from the CCRC.

Children participating in the PHiD-CV clinical trial were included in the memory B cell sub-study if they were 12-24 months old and if they consented to take part in the PRISM study.

2.5 Criteria for exclusion of study participants

Any child whose guardian did not give written informed consent or withdrew consent to participate in the study was excluded. In addition, HIV-infected children at CCRC were excluded if:

- They had severe malnutrition i.e. mid upper arm circumference <11cm.
- They had any other active infection e.g. tuberculosis.

Community controls from the malaria surveillance cohort were excluded if:

- They had an acute illness at the time of sampling or in the previous week.
- They had a positive malaria slide.

Children participating in the PHiD-CV clinical trial were excluded if:

- They had febrile illness (temperature >38.5°C axillary) at the time of vaccine dose 1.
- They had previously received any pneumococcal vaccine.
- They had previously received DTP-containing vaccine after the first year of life.
- They had severe malnutrition (mid upper arm<11.5 cm) or other serious medical conditions (e.g. AIDS, tuberculosis, malignancy).
- They had convulsions in the past 6 months.
- They had known allergies to vaccines or vaccine components.
- They were resident in Kilifi Demographic Surveillance area.
- They intended to leave the study area in the following six months.

2.6 Sampling procedures

At the CCRC, study participants were asked to donate additional 3 ml of blood in heparin tubes for the study at the same time when they gave 2 ml in Ethylene Diamine Tetraacetic Acid (EDTA) tubes for routine monitoring of their $CD4^+$ T-cell counts. This reduced the distress associated with being bled. They were also asked if the left over blood from the $CD4^+$ T-cell count could be used for the study.

Community controls donated 5ml of blood in heparin tubes during the annual cross-sectional survey that is part of the malaria surveillance studies.

Participants of the PRISM clinical trial donated 5 ml of blood in heparin tubes during each of the scheduled time points as per the protocol of the trial.

Trained phlebotomists, nurses or clinical officers did all collection of blood samples.

In the collection of nasopharyngeal swabs, a rayon-tipped nasal swab was passed along the nasal-cavity floor to the posterior wall of the nasopharynx where it was left for two seconds then rotated 180° before being removed. The swabs were then placed in skim-milk tryptone glucose glycerol (STGG) transport media and processed within 8 hours. Optochin susceptibility testing and Quellung serotyping were used to identify Pneumococci from gentamicin-blood agar.

2.7 Processing of samples in the laboratory

Once collected, the blood samples were transported to the laboratory in cooler boxes. In the laboratory, the EDTA sample was used for complete blood counts and $CD4^+$ T-cell counts determination. It was then used for whole blood flow cytometry staining. Plasma was separated from the left over EDTA sample and stored at -20°C for later determination of plasma viral load. For cultured B-cell ELISpots, the heparin tube

samples were used. First, plasma was separated from the cellular fraction by centrifugation and stored at -20 °C for later determination of plasma antibodies and cytokines. The cellular fraction was then re-suspended in culture media and PBMCs isolated by density gradient centrifugation using Lymphoprep®. The freshly isolated PBMCs were then cultured for ELISpots to determine the frequencies of antigen specific memory B cells.

Buffy coats were suspended in RPMI and subjected to density gradient centrifugation for isolation of PBMCs. Some of the cells were stored in liquid nitrogen for later in vitro experiments.

2.8 Determination of $CD4^+$ T cell counts and complete blood counts

Determination of $CD4^+$ T-cell counts and complete blood counts was outsourced from the KEMRI-Wellcome Trust Research Programme Clinical Trials Laboratory (CTL). $CD4^+$ T-cell counts were done using BD FACSCount™ machine. Complete blood counts were done using Beckman Coulter Ac.T 5 diff CP machine.

2.9 Determination of plasma viral loads

Determination of plasma viral loads was outsourced from the International Centre for Reproductive Health (ICRH) laboratories in Mombasa. They used the Generic HIV Viral Load assay, (Biocentric) – a RT-qPCR test developed by the Agence Nationale de Recherches sur le SIDA (ANRS) targeting a well-conserved LTR region [218, 219].

2.10 Cell preparation and staining of surface markers for determination of phenotypes of B cells in children infected with HIV

Frequencies of the lymphocyte subsets were determined in whole blood by multi-colour flow cytometry. The following antibodies were used: anti-CD19-ECD, anti-CD3-ECD, anti-CD27-Pe-Cy5, anti-CD10-FITC, anti-IgD-PE, anti-CD21-FITC (Beckman Coulter); anti-CD3-FITC, anti-CD4-Pe-Cy7, anti-CD8-APC-H7, anti-CD20-APC-H7, anti-CD10-PE (BD Biosciences); anti-CD21-PE and anti-CD38-Pe-Cy7(eBioscience).

Whole blood was washed twice with Phosphate Buffered Saline containing 2% Fetal Calf Serum (FCS), 5mM EDTA and 1% Sodium Azide. It was then incubated with staining antibodies for 30 minutes, RBCs lysed for 10 minutes in OptiLyse C® lysis Solution (Beckman Coulter) and washed again before analysis. A no-wash staining procedure in Trucount tubes (BD Biosciences) was done to determine absolute counts of T cells and B cells. A nine-colour Cyan ADP flow cytometer (Beckman Coulter) was used. The data were analysed using Flowjo version 9.2 (TreeStar).

2.11 ELISA for determination of quantities of antibodies to vaccines and common childhood infections

Plasma samples were tested for IgG and IgM against vaccine antigens using a previously established ELISA protocol with various modifications [220]. Briefly, plates were coated overnight at 4°C with 100 µL/well antigen solution (1µg/ml for tetanus toxoid and diphtheria toxoid, 5µg/ml for measles antigen) in bicarbonate buffer. Tetanus toxoid and diphtheria toxoid were obtained from Statens Serum Institut whereas the measles virus lysate/antigen was obtained from Meridian Life sciences. For

determination of antibodies against pneumococcal capsular polysaccharides, a mixture of capsular polysaccharides from ATCC (serotypes 1, 5, 6B, 14, 19F and 23F, each at 10 μg/ml) in PBS was used. The plates were then washed three times with the wash buffer (0.05% tween in PBS) followed by blocking for at least 1 hour with 200 μL/well of blocking buffer (10% Foetal Calf Serum in PBS). After discarding the blocking buffer, the plates were incubated with 100 μL/well of the test plasma samples at a dilution of 1:200 in the sample diluent buffer (0.3% tween in PBS +EDTA) for two hours. They were then washed four times and incubated with 100 μL/well HRP-conjugated donkey antihuman IgG/IgM diluted at 1:5000 in the conjugate diluent buffer (0.05% tween in PBS). After one hour, the plates were washed five times and the HRP labelled conjugate revealed with 100 μL/well OPD substrate (Sigma). The substrate reaction was stopped by adding 50 μL/well of 2N sulphuric acid. The optical density was determined at 490 nm. Antibodies were quantified against hyper-immune plasma that was used to generate a standard curve for each plate. Antibody concentrations were expressed as arbitrary units that were calculated against the standard curve for the respective plates.

For the PRISM study, the determination of IgG against each of the 10 vaccine serotypes was done by other scientists in the WHO Pneumococcal Reference Laboratory at Institute of Child Health, University College, London.

2.12 Modified ELISA for determination of avidity of antibodies to vaccines and common childhood infections

For determination of the avidity of IgG antibodies, the above ELISA method was used with some modifications. After incubating with the test plasma samples, the bound antibodies were eluted by incubating the plates with 100 μL/well of a chaotropic agent

(2M Guanidine Hydrochloride in conjugate buffer) for ten minutes. The control wells for each sample (on the same plate) were incubated with the conjugate buffer. The plates were then incubated with HRP-conjugated donkey antihuman IgG and developed as described above. The avidity index was calculated as the ratio of the quantity of antibodies in the eluted wells to the quantity in the control non-eluted wells.

2.13 Cultured B-cell ELISpot for determination of frequencies of antigen specific memory B cells

Memory B cells against vaccine antigens were quantified using the standard method developed by Crotty et al [221]. Briefly, freshly isolated PBMCs were cultured for 6 days in RPMI complete media (RPMI + 10% newborn bovine serum + L-glutamine + penicillin Streptomycin + Hepes + β-mercaptoethanol) containing 2.5 μg/ml CpG ODN 2006 (Hycult Biotech), 1:5000 dilution of *Staphylococcus aureus* Cowan Strain protein A (Sigma) and 0.083 μg/ml of Poke-Weed Mitogen (Sigma) at $2x10^5$ cells per 200 μL in U-bottomed 96-well culture plates.

ELISpot plates were pre-coated overnight at 4°C with the antigen of interest (at a concentration of 5μg/ml for tetanus toxoid or 10μg/ml for measles antigen) or with Goat antihuman Igs (Caltag) at 10μg/ml. Negative control wells were coated with 1% BSA as a non-specific protein control. For determination of memory B cells against pneumococcal capsular polysaccharides in CCRC and Ngerenya children, a mixture of capsular polysaccharides from ATCC (serotypes 1, 5, 6B, 14, 19F and 23F, each at 10 μg/ml) conjugated with 30 μg/ml of methylated human serum albumin (mHSA) (National Institute for Biological Standards and Control-UK) was used. For determination of serotype-specific memory B-cell responses in children who

participated in the PRISM study, pneumococcal capsular polysaccharides for serotypes 1 (20 μg/ml), 6B (10 μg/ml), 14 (10 μg/ml), 19F (20 μg/ml) and 23F (20 μg/ml) obtained from GSK biologicals, Rixensart, Belgium were used separately and were conjugated to mHSA at a concentration of 10 μg/ml for serotypes 1 & 14 and 20 μg/ml for serotypes 6B, 19F & 23F. Negative control wells were coated with mHSA at 10 μg/ml.

The ELISpot plates were washed three times with PBS and blocked for at least one hour with 10% New Born Bovine Serum (NBBS) in RPMI. PBMCs harvested from the 6-day cultures were seeded into the ELIPSpot plates at 200 and 2000 cells /well (total IgG secreting cells) and at 2 x 20^5 cells/well (antigen-specific IgG secreting cells) and incubated at 37°C in 5% carbon dioxide and 95% humidity overnight. The cells were discarded and the plates were washed four times with 0.25% tween in PBS followed by a five-minute soak with PBS. They were then incubated with 50 μL/well of Alkaline phosphatase conjugated donkey antihuman IgG (Jackson ImmunoResearch Laboratories) for two hours followed by five washes with 0.25% tween in PBS and three washes in distilled water. Spots were developed by adding 50μL/well of substrate (Bio-Rad). The reaction was stopped by washing with distilled water. The plates were left to dry, then scanned using CTL Immunospot analyser. The spots were counted using pre-established settings in Immunocapture software.

2.14 Staining PBMCs with carboxyfluorescein diacetate succinimidyl ester (CFSE) for determination of proliferation in culture

Tracking of proliferation of PBMCs was done using the CellTrace™ CFSE Cell Proliferation Kit (Invitrogen) in accordance to the manufacturer's instructions. Briefly,

the cells were suspended in prewarmed PBS/0.1% BSA at a final concentration of $1X10^6$ cells/ml. CFSE dissolved in anhydrous DMSO was then added to the cells to give a final working concentration of 1μM. The cells' suspension was mixed thoroughly and incubated at 37°C for 10 minutes. The staining was quenched by adding 5 volumes of ice-cold culture media to the cells followed by incubation in ice for 5 minutes. The cells were then pelleted by centrifugation, resuspended and washed twice before being placed in culture.

2.15 Setting up of cultures for determination of in vitro effects of various B-cell stimulants and the effects of recombinant HIV-1 Nef on the B-cell responses to the stimulants.

PBMCs isolated from buffy coats donated by healthy donors were cultured at $2x10^5$ cells per 200 μL in U-bottomed 96-well culture plates at 37°C in 5% carbon dioxide and 95% humidity. In some cases, stored PBMCs were thawed rapidly at 37°C, washed and counted then cultured. Some of the cultures were set up using CFSE-stained cells for tracking of proliferation in culture.

In experiments where the effect of HIV-1 Nef (Jena Bioscience) was being evaluated, cells were first pre-incubated with recombinant HIV-1 proteins (100 ng/ml of wild-type HIV-1 Nef or mutant non-myristoylated HIV-1 Nef) or media alone for 24 hours.

The various stimulant-combinations were then added at pre-determined concentrations: CpG at 1μg/ml, R848 at 100 ng/ml, CD40L at 125 ng/ml (with enhancer for ligand at 250 ng/ml), anti-Igs (BCR ligand) at 1.25μg/ml and IL21 at 50 ng/ml. Supernatants and cells were harvested from some of the wells 48 hour after addition of stimulants. The supernatants were used for determination of secretion of IL6 and TNF-alpha while the

cells were used for determination of expression of activation markers on B cells. Five days after addition of stimulants, cells were harvested from some wells for determination of proliferation and differentiation into plasmablasts. Eight days after addition of stimulants, culture supernatants were harvested from the remaining wells and used for determination of secretion of immunoglobulins.

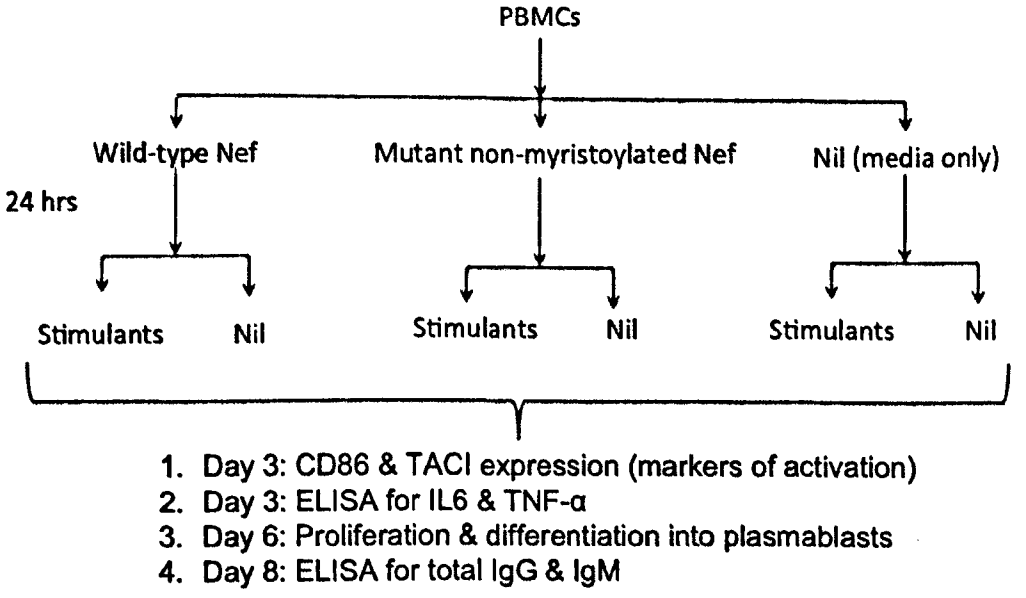

Figure 2.3: Experimental design for determination of in vitro effects of recombinant HIV-1 Nef. The various assays used to evaluate B-cell responses have been listed.

2.16 Flow cytometry for determination of proliferation, differentiation into plasmablasts and expression of activation markers in cultured cells

Anti-CD19-Brilliant Violet 421™ (Biolegend) was used for identification of B cells while Aqua LIVE/DEAD ® Fixable dead Cell Stain kit (Invitrogen) was used to

identify live cells. For determination of expression of activation markers, anti-CD86-PE (Biolegend) and anti-TACI-PE (Biolegend) were used. Differentiation of B cells into plasmablasts was assessed using anti-CD38-PeCy7 (Biolegend) and anti-CD27-PeCy5 (Beckman Coulter). Proliferating cells were identified based on their content of CSFE stain.

Harvested cultured cells were stained for viability in accordance with the Aqua LIVE/DEAD stain Aqua LIVE/DEAD ® Fixable dead Cell Stain manufacturer's instructions. Briefly, the cells were washed once in PBS and resuspended in PBS at a concentration of $2x10^5$ cells per 200µL. 0.2µL of the viability stain was added and the mixture was vortexed thoroughly. The sample was incubated on ice, protected from light, for 30 minutes. The cells were then washed in 1 ml PBS and resuspended.

To stain for expression of surface markers, the cells were incubated with staining antibodies for 30 minutes, washed in FACS buffer and acquired in an eleven-colour LSR II flow cytometer (BD). The data were analysed using Flowjo version 9.2 (TreeStar).

2.17 ELISA for determination of total immunoglobulins secreted into culture supernatants

Culture supernatants were tested for total IgG and IgM using the protocol for determination of antigen specific antibodies with various modifications. Briefly, plates were coated overnight at room temperature with Goat antihuman Igs (Caltag) at 10µg/ml in PBS (100 µL/well). They were then washed three times with the wash buffer (0.05% tween in PBS) followed with blocking for at least 1 hour with 200 µL/well of

blocking buffer (1% BSA in PBS). After discarding the blocking buffer, the plates were washed 3 times and incubated with 50 μL/well of the test culture supernatants at a dilution of 1:4 in the reagent diluent buffer (1% BSA in PBS) for two hours. They were then washed three times and incubated with 50μL/well HRP-conjugated donkey antihuman IgG/IgM (Jackson Immunoresearch laboratories) diluted at 1:5000 in the reagent diluent (1% BSA in PBS). After one hour, the plates were washed three times and the HRP labelled conjugate revealed with 100 μL/well OPD substrate (Sigma). The substrate reaction was stopped by adding 50 μL/well of 2N sulphuric acid. The optical density was determined at 490 nm. Antibodies were quantified against purified human IgG/IgM (Sigma) that was used to generate a standard curve for each plate.

2.18 ELISA for determination of cytokines secreted into culture supernatants

Determination of TNF-∝ in culture supernatants was done using the DuoSet® ELISA system (R&D systems) in accordance with the manufacturer's instructions. First, 96-well microplates were coated/incubated with 50μL of capture antibody solution, mouse anti-human TNF-∝, at 4μg/ml in PBS overnight at room temperature. They were then washed three times with the wash buffer (0.05% tween-20 in PBS). They were subsequently blocked by adding 200 μL of the reagent diluent (1% BSA in PBS) for at least 1 hour. After discarding the reagent diluent, the plates were washed thrice and incubated with 50μL of the culture supernatants diluted at 1:2 in the reagent diluent. They were then incubated for two hours at room temperature. The wash (three times with wash buffer) was repeated followed by incubation with 50μL of detection antibody, biotinylated goat anti-human TNF-∝, at a concentration of 500ng/ml in reagent diluent. The plates were incubated for a further two hours at room temperature,

washed three times with wash buffer and incubated with 50μL of 1:200 streptavidin-HRP solution in darkness at room temperature for 20 minutes. They were then washed with wash buffer three times. 100μL of OPD substrate (Sigma) solution was added and left for 10 minutes away from light for the colour to develop. The reaction was stopped by adding 50 μL of 2N sulphuric acid. The optical density was determined at 490 nm. TNF-∝ levels were quantified against recombinant human TNF-∝ that was used to generate a standard curve.

Determination of IL6 in culture supernatants was also done using the DuoSet® ELISA system (R&D systems) in accordance with the manufacturer's instructions. The procedure was the same as that used for determination of TNF-∝ levels but with some modifications. The capture antibody used was mouse anti-human IL6 (at 2μg/ml) while the detection antibody used was biotinylated goat anti-human IL6 (at 50ng/ml). The standard curve was generated using recombinant human IL6.

2.19 Data storage

The data were stored in personal computers and backed up in a central server and external hard disks.

2.20 Statistical analysis

The data were analysed using STATA version 11.0 (STATA Corporation TX, USA). P values below 0.05 were considered to be significant. Since the data were skewed, comparisons between groups were done using non-parametric tests. In particular, Wilcoxon rank-sum test was used to compare groups of children that were not paired

whereas Wilcoxon sign-rank test was used to compare paired samples from the same children that were collected at different time points. Regression analyses were done using quantile regression models. Stata Predxcon package (using linear regression models) was used to test for statistical interactions. Spearman's correlations were done to assess correlations between various variables.

Chapter 3 Phenotype of B cells in children infected with HIV

3.1 Literature review

Although HIV primarily targets $CD4^+$ T cells, it has been shown to affect all other lymphocyte populations, including B cells. In fact, defects in the B-cell compartment were among the first to be described in the early days of the epidemic [122]. Later studies showed that B cells in HIV patients over-express markers of activation and are terminally differentiated [123, 222].

The effect of ongoing viral replication in viraemic patients reflects a combination of both direct interactions of B cells with the virus and indirect interactions that are associated with a wide range of systemic alterations. There is little evidence to suggest that HIV can productively replicate in B cells. However, B cells in HIV patients' peripheral blood and lymphoid tissue have been shown to bind virions through CD21-complement interactions and transmit infectious virus to activated T cells [223]. They therefore act as extracellular reservoirs like follicular dendritic cells, but with the added capability of circulating in peripheral blood and migrating through tissues where they can potentially interact with and pass the virus to T cells. Other surface receptors that interact with HIV virions have been described in B cells. DC-specific ICAM 3-grabbing non-integrin (DC-SIGN) has been described as a portal on a subset of B cells for HIV-1 infection of T cells [224]. It has also been demonstrated that a subset of B cells binds gp120 through mannose C-type lectin receptors (MCLRs), leading to polyclonal class switch recombination through a CD40-independent mechanism [132]. In another study, B cells that express VH3 family Immunoglobulin were shown to polyclonally bind HIV gp120 by means of membrane immunoglobulin. The gp120 selectively induced immunoglobulin secretion by VH3 B cells, indicating that the binding of gp120 functionally activated these cells. These results indicate that naturally occurring VH3 immunoglobulin is a ligand for gp120 and that gp120 is a candidate superantigen for VH3 B cells [225]. Of note, the direct effects of HIV virions on B cells described above

have only been demonstrated in vitro. Furthermore, the fraction of B cells carrying HIV virions on their surface is relatively low compared to the extent of B-cell dysfunction, suggesting that other indirect systemic effects may play a major role.

Recent introduction of HAART has enabled delineation of the immune defects associated with HIV infection that are related to ongoing viral replication as opposed to defects that persist despite the suppression of detectable viraemia. Additionally, technological advancement with detailed characterization of B-cell subpopulations in health and disease has allowed scientists to better understand the mechanisms of pathogenesis of HIV by comparing B-cell subset distribution in patients before and after control of viraemia.

In healthy adults, most B cells in the peripheral blood are either mature naive B cells or resting memory B cells. These two populations express high levels of CD21 (CR2). However, in viraemic HIV-infected adults, there is expansion of a CD21 low subset that appears to contract to normal levels upon control of viraemia with HAART [123]. This CD21 low subset has been shown to be heterogeneous, consisting of smaller subsets that are themselves independently expanded in the viraemic state.

The CD21 low immature/transitional B cells ($CD19^+$, $CD10^+$, $CD21^{low}$, $CD38^{++}$) are particularly increased in these patients. Their frequencies in adults have been shown to negatively correlate with $CD4^+$ T-cell counts, implying that $CD4^+$ T-cell lymphopenia drives this expansion and not viraemia per se. IL7, a T cell homeostatic cytokine, has been suggested to play a major role in causing this defect [226].

Another CD21 low B cell subset ($CD19^+$, $CD10^-$, $CD21^{low}$, $CD27^-$) has been recently described and is also expanded in viraemic patients. It comprises of cells that show properties of memory B cells i.e. some degree of somatic hypermutation and class switching as well as some replication history despite lacking CD27 expression, the

classical marker of human memory B cells. These cells also express a range of inhibitory markers and homing receptors that favour migration to inflammatory sites away from lymphoid organs. Their phenotypic profile is similar to that observed in B cells isolated from tonsils of patients undergoing tonsillectomy [227].

Other CD21 low B-cell subsets that are elevated in HIV viraemic adults are plasmablasts ($CD19^{+}$, $CD21^{low}$, $CD20^{low}$, $CD27^{++}$, $CD38^{+++}$) and activated mature B cells ($CD19^{+}$, $CD21^{low}$, $CD27^{+}$). Their frequencies have been shown to increase several fold in viraemic states and resolve after initiation of HAART [123, 215].

Notably, HIV infection in adults depletes the resting memory B-cell compartment ($CD19^{+}$, $CD21^{high}$, $CD27^{+}$) [228]. This depletion has been shown to occur early in infection. Initiation of HAART does not restore this compartment. The mechanism of the depletion amidst generalized immune activation is poorly understood. It has been shown to correlate with the subsequent reduction of serum antibodies to non-HIV antigens and may contribute to the observed impairment of the humoral immune responses in HIV-infected patients [229].

The above B-cell defects have been mainly described in adults with HIV. Children infected with HIV differ from adults in that their immune system is immature and develops against a background of HIV infection while adults get infected much later in life when their immune system has already matured. Additionally, children get infected when they have already been exposed to some HIV factors in-utero while adults get infected when they are totally naive to exposure to such HIV factors. Exposure of children to activating cytokines from their mothers in-utero could also modulate the interaction of their immune system with the virus.

Much less is known about the subset distribution of B cells in children infected with HIV. One study was conducted in older children receiving HAART and showed that

despite effective HAART, these children had reduced frequency of IgD^+ memory while IgD^- memory B-cell numbers were not affected [230]. On the other hand, another study indicated a reduction in both switched and unswitched memory B cells that correlated with viral load [231]. An additional study investigated the effect of early initiation of HAART. It reported that initiation of HAART in the first year of life preserved the memory B-cell compartment while later initiation, regardless of the resultant control of viraemia, led to depletion of memory B cells [232]. Notably, the above studies limited their investigation on the memory B-cell compartments. A later study investigated the effect of active HIV viraemia on immature/transitional B cells in children. Unlike healthy and aviraemic children, viraemic children showed a lack of age-related contraction of the immature/transitional B-cell compartment [233]. A more detailed and recent study showed that HIV-infected children who have very low $CD4^+$ T-cell percentages have reduced frequencies of naive B cells and resting memory B cells while their activated subsets are expanded [234].

Noteworthy is the fact that determination of B-cell subset distribution in children has not been done to the same depth as in adults.

3.2 Objective:

To determine the subset distribution of B cells in children infected with HIV.

3.3 Gating strategy and data analysis

Samples were prepared and acquired on the flow cytometer as described in chapter 2.10.

The data were analysed using Flowjo version 9.2 (TreeStar). Lymphocytes were gated on the forward versus side scatter plot. Naïve B cells were defined as $CD19^+$ $CD27^-$ $CD21^+$, resting memory B cells were defined as $CD19^+$ $CD27^+$ $CD21^+$, activated mature

B cells were defined as $CD19^+$ $CD27^+$ $CD21^-$, atypical memory B cells were defined as $CD19^+$ $CD27^-$ $CD21^-$, plasmablasts were defined as $CD19^+$ $CD27^{++}$ $CD38^{+++}$, immature/transitional B cells were defined as $CD19^+$ $CD10^+$ $CD38^{++}$, IgD^+ (unswitched) resting memory B cells were defined as $CD19^+$ $CD27^+$ $CD21^+$ IgD^+ and IgD^- (switched) resting memory B cells were defined as $CD19^+$ $CD27^+$ $CD21^+$ IgD^-. For the determination of absolute counts of lymphocyte lineage populations, B cells were defined as $CD19^+$, $CD4^+$ T cells were defined as $CD3^+$ $CD4^+$ $CD8^-$ while $CD8^+$ T Cells were defined as $CD3^+$ $CD4^-$ $CD8^+$.

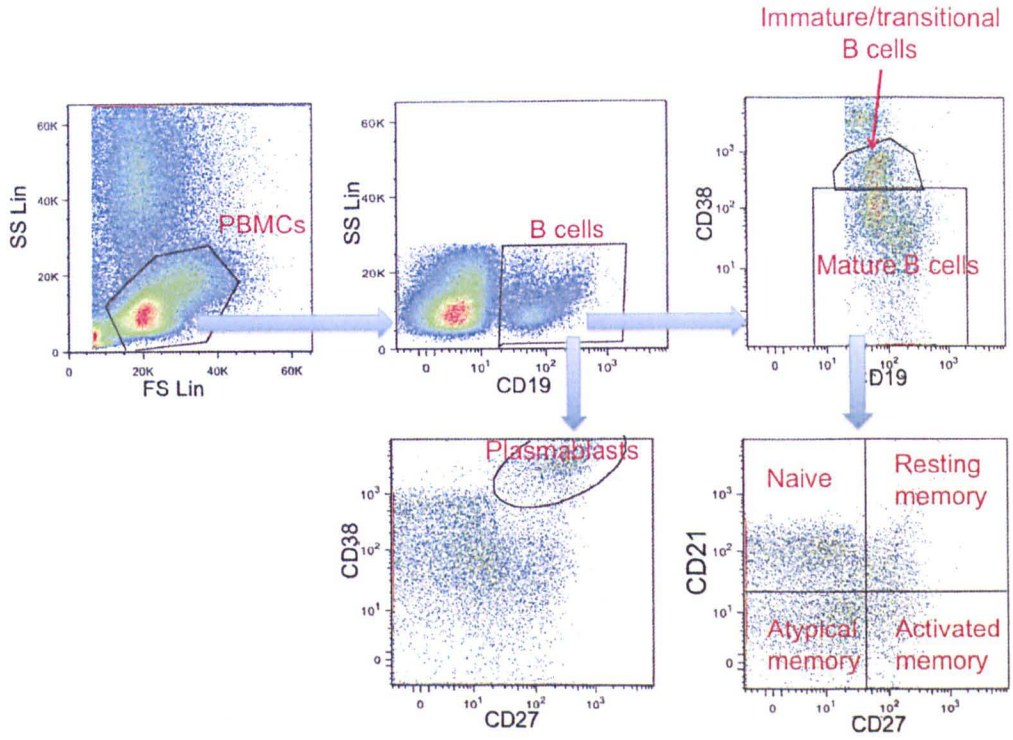

Figure 3.1: Gating strategy to identify the different B-cell subsets in peripheral blood from a HIV-infected child with high viraemia. Lymphocytes were identified on forward scatter-side scatter plot. Within the lymphocytes gate, B cells were identified based on the expression of CD19. Subsequent identification of B-cell subsets was done within the B-cell gate.

3.4 Results

3.4.1 General population characteristics and frequencies of various lymphocyte lineages

A total of 78 HIV-infected children were recruited into the study. 36 (46 %) children had high viraemia (>=5000 RNA copies per ml) while 42 (54 %) children had low viraemia (<5000 RNA copies per ml). A further 28 children were recruited from a low-endemicity malaria surveillance cohort as community controls. The population characteristics are as shown in table 3.1.

As would be expected, the HIV-infected children had lower $CD4^+$ T-cell percentages (25.5% [IQR, 17.8%-32.1%]) when compared with the community control children (33.8% [IQR, 28.1%-39.3%] P=0.0001). In addition, the high viraemia group had $CD4^+$ T-cell percentages (22.2% [IQR, 9.2%-26.8%]) that were significantly lower than those of both the community controls (33.8% [IQR, 28.1%-39.3%] P<0.00005) and the low viraemia group (29.4% [IQR, 22.5%-34.6%] P=0.0026). Notably, the low viraemia group had $CD4^+$ T-cell percentages that were lower than the community controls (P=0.0102). 22 (61%) highly viraemic children had low $CD4^+$ T-cell percentages while 14 (33%) of children with low viraemia had low $CD4^+$ T-cell percentages. Consequently, among the HIV-infected children, there was an inverse correlation between viraemia and $CD4^+$ T-cell percentages (rho=-0.4454, P=0.0001).

Further stratification of the low viraemia group showed that those treated with HAART for at least 6 months also had lower percentages of $CD4^+$ T-cells when compared with community controls (29.8% [IQR, 25.5%-36.3%] P=0.0446), suggesting that they had only experienced partial recovery of $CD4^+$ T-cell percentages despite effective control of viraemia upon initiation of HAART. Similarly, the HAART naïve children who had low viraemia had lower frequencies of $CD4^+$ T cells when compared with community

controls (23.0% [IQR, 20.4%-33.4%] P=0.0125), suggesting that they had lost $CD4^+$ T cells despite maintaining low viraemia. Their $CD4^+$ T-cell percentages were also similar to those of HAART naïve children who had high viraemia (18.9% [IQR, 8.8%-27.4%] P=0.2048) and HAART-receiving children who had high viraemia (23.1% [IQR, 13.0%-26.6%] P=0.4009). Notably, the children who had low viraemia and were on HAART had higher $CD4^+$ T-cell percentages when compared with those who had high viraemia without HAART (P=0.0039), confirming that control of viraemia after initiation of HAART leads to some recovery of $CD4^+$ T-cell percentages. There were no differences in $CD4^+$ T-cell percentages when HAART-treated children were compared with HAART-naïve children within both high viraemia and low viraemia groups.

B-cell percentages were lower in the HIV-infected children (16.5% [IQR, 11.1%-20.9%]) when compared with community controls (20.5% [IQR, 15.7%-23.7%] P=0.0106). This effect was also observed in the low viraemia group (15.3% [IQR, 11.2%-20.7%] P=0.0091). On the other hand, the high viraemia group only had a trend towards having low B-cell percentages (17.8% [IQR, 9.8%-22.2%] P=0.0611) when compared with community controls. Further stratification of the low viraemia group revealed that the children who were on HAART had lower B-cell percentages (15.0% [IQR, 11.8%-19.5%]) when compared with community controls (P=0.0113). However, children who had low viraemia and were HAART naïve did not differ from community controls in their B-cell percentages. HAART-naïve HIV-infected children were similar with regard to their B-cell percentages regardless of their levels of viraemia. Similarly, children who were HAART-naïve and had high viraemia were comparable to children who were on HAART and had low viraemia, implying that controlling viraemia after initiating HAART had not caused a difference in percentages of total B cells. Further

comparison of HAART-receiving versus HAART-naïve children did not reveal any differences in B-cell percentages within both high viraemia and low viraemia groups.

When the children were stratified on the basis of their $CD4^+$ T-cell percentages, children who had low $CD4^+$ T-cell percentages showed lower frequencies of B cells (15.2% [IQR, 9.6%-19.8%]) when compared with the community controls (20.5% [IQR, 15.7%-23.7%] P=0.0048). Upon further stratification on the bases of HAART treatment, both HAART-naïve and HAART-treated children with low $CD4^+$ T-cell percentages had lower frequencies of B cells (15.2% [IQR, 10.3%-20.9%], P=0.0270 and 16.0% [IQR, 9.4%-19.3%], P=0.0106 respectively) when compared with community controls, suggesting that immunological failure correlated with depletion of total B cells. Interestingly, children who were HAART naïve but had high $CD4^+$ T-cell percentages were comparable with community controls, implying that maintenance of $CD4^+$ T-cell frequencies in absence of HAART is associated with maintenance of total B-cell frequencies. However, children who had high $CD4^+$ T-cell percentages and were on HAART had lower frequencies of B cells when compared with community controls (15.8% [IQR, 12.1%-19.5%] versus 20.5% [IQR, 15.7%-23.7%], P=0.0180), suggesting that initiation of HAART and subsequent restoration of $CD4^+$ T-cell frequencies was not associated with restoration of B-cell frequencies.

$CD8^+$ T-cell percentages were elevated in the whole HIV-infected cohort when compared with the community controls (32.8% [IQR, 25.2%-41.7%] versus 15.8% [IQR, 13.7%-17.0%] P<0.00005). Both high and low viraemia groups had higher $CD8^+$ T-cell percentages (37.1% [IQR, 31.5%-44.9%] and 27.0% [IQR, 22.2%-39.0%] respectively) than the community controls (15.8% [IQR, 13.7%-17.0%] P<0.00005 in both cases). In addition, the high viraemia group had higher $CD8^+$ T-cell percentages than the low viraemia HIV-infected group (P=0.0003). When the children were further stratified on the basis of their HAART-treatment status, there were no differences

between HAART-treated and HAART naïve children in both high viraemia group (37.2% [IQR, 31.8%-43.9%] versus 35.4% [IQR, 29.4%-50.4%] P=0.8684) and low viraemia group (25.5% [IQR, 16.6%-38.4%] versus 29.4% [IQR, 24.5%-40.7%] P=0.2589) with regard to $CD8^+$ T-cell percentages. Furthermore, both HAART-treated and HAART-naïve children with high viraemia had higher percentages of $CD8^+$ T cells when compared with HAART naïve children who had low viraemia (P=0.0040 and 0.0298 respectively). HAART-naïve children who had high viraemia also had higher frequencies of CD8+ T cells when compared with HAART-receiving children who had low viraemia (P=0.0033), suggesting that control of viraemia after initiation of HAART leads to some recovery of the $CD8^+$ T-cell compartment. In addition, children with low viraemia, regardless of HAART treatment status, had higher frequencies of $CD8^+$ T-cell frequencies when compared with community controls (P<0.00005 for HAART-naïve children and P=0.0017 for HAART-treated children), suggesting that control of viraemia only partially normalizes $CD8^+$ T-cell percentages.

Similar effects were observed when the children were stratified on the basis of their immunological status. Children who had low $CD4^+$ T-cell percentages showed higher frequencies of $CD8^+$ T-cell frequencies (39.4% [IQR, 32.1%-43.9%]) when compared with children that had high $CD4^+$ T-cell percentages (29.2% [IQR, 24.6%-36.0%] P<0.00005) and community controls (15.7% [IQR, 13.7%-17.0%] P<0.00005). Even after further stratification on the bases of HAART treatment, the HAART-naïve and HAART-treated children, regardless of their $CD4^+$ T-cell percentage status, had higher frequencies of $CD8^+$ T cells (38.4% [IQR, 25.4%-43.9%] and 40.7% [IQR, 34.7%-47.6%] for children with low $CD4^+$ T-cell percentage and 30.7% [IQR, 26.4%-38.1%] and 26.6% [IQR, 23.3%-32.4%] for children with high $CD4^+$ T-cell percentage) when compared with community controls (P<0.00005 in all cases), suggesting that immunological recovery does not cause full restoration of normal $CD8^+$ T-cell

frequencies and maintenance of high $CD4^+$ T-cell frequencies does not preserve the state of the $CD8^+$ T-cell compartment. However, immunological recovery upon initiation of HAART was associated with partial recovery in the $CD8^+$ T-cell compartment as children on HAART and having high $CD4^+$ T-cell percentages had lower frequencies of $CD8^+$ T cells when compared with HAART-naïve children who had low $CD4^+$ T-cell percentages (P=0.0164).

Interestingly, 13 of the 38 HAART naïve children had viral loads below 5000 RNA copies/ml, implying that some children were able to control viraemia naturally. In addition, 11 of the 40 HAART receiving children had virological failure (viral loads above 5000 RNA copies/ml despite HAART), suggesting that a significant proportion of children could be unable to control viral replication despite being on HAART for at least six months.

	Value	P value (versus community controls)
Number (N)	106	
Community controls	28	
HIV-infected	78	
Low viraemia	42	
High viraemia	36	
High $CD4^+$ T-cell percentage	36	
Low $CD4^+$ T-cell percentage	42	
Median age in months	49 (31-74)	
Community controls	56 (39-86)	
HIV-infected	46 (26-71)	0.0656 [a]
Low viraemia	46 (25-80)	0.1861 [a]
High viraemia	43 (31-60)	0.0503 [a]
High $CD4^+$ T-cell percentage	48 (31-72)	0.8054 [a]
Low $CD4^+$ T-cell percentage	38 (27-69)	0.0209 [a]
% Female (n)	53 (54)	
Community controls	54 (15)	
HIV-infected	53 (41)	0.937 [b]
Low viraemia	47 (17)	0.748 [b]
High viraemia	59 (24)	0.610 [b]
High $CD4^+$ T-cell percentage	61 (25)	0.541 [b]
Low $CD4^+$ T-cell percentage	42 (14)	0.385 [b]
% on HAART(n)	N/A	
Community controls	N/A	
HIV-infected	51 (40)	
Low viraemia	69 (29)	
High viraemia	31 (11)	
High $CD4^+$ T-cell percentage	62 (26)	
Low $CD4^+$ T-cell percentage	39 (14)	
Viral load, log10 RNA copies/mL	N/A	
Community controls	N/A	
HIV-infected	3.4 (0.18-4.78)	
Low viraemia	1.25 (0.0-3.2)	
High viraemia	4.8 (4.4-5.3)	
High $CD4^+$ T-cell percentage	2.7 (0.0-4.1)	
Low $CD4^+$ T-cell percentage	4.6 (3.2-5.0)	
$CD4^+$ T cells (Percentage)	27.6 (21.1-34.5)	
Community controls	33.8 (28.1-39.3)	
HIV-infected	25.5 (17.8-32.1)	0.0001 [a]
Low viraemia	29.4 (22.5-34.6)	0.0102 [a]

High viraemia	22.2 (9.2-26.8)	<0.00005 [a]
High $CD4^+$ T-cell percentage	31.9 (27.9-37.8)	0.3377 [a]
Low $CD4^+$ T-cell percentage	17.1 (8.6-22.5)	<0.00005 [a]
$CD8^+$ T cells (Percentage)	27.2 (16.9-39.3)	
Community controls	15.8 (13.7-17.0)	
HIV-infected	32.8 (25.2-41.7)	<0.00005 [a]
Low viraemia	27.0 (22.2-39.0)	<0.00005 [a]
High viraemia	37.1 (31.5-44.9)	<0.00005 [a]
High $CD4^+$ T-cell percentage	29.2 (24.6-36.0)	<0.00005 [a]
Low $CD4^+$ T-cell percentage	39.4 (32.1-43.9)	<0.00005 [a]
B cells (Percentage)	17.9 (12.4-22.5)	
Community controls	20.5 (15.7-23.7)	
HIV-infected	16.5 (11.1-20.9)	0.0106 [a]
Low viraemia	15.3 (11.2-20.7)	0.0091 [a]
High viraemia	17.8(9.8-22.2)	0.0611 [a]
High $CD4^+$ T-cell percentage	17.3 (12.4-23.3)	0.0777 [a]
Low $CD4^+$ T-cell percentage	15.2 (9.7-19.8)	0.0048 [a]
Hemoglobin, g/dL	11.1(9.9-11.9)	
Community controls	11.9 (11.4-12.3)	
HIV-infected	10.6 (9.6-11.6)	0.0001 [a]
Low viraemia	11.3 (10.2-12.0)	0.0217 [a]
High viraemia	10.1 (9.1-11.1)	<0.00005 [a]
High $CD4^+$ T-cell percentage	11.3 (10.1-12.1)	0.0340 [a]
Low $CD4^+$ T-cell percentage	10.0 (8.8-11.0)	<0.00005 [a]

Table 3.1: Baseline characteristics of the HIV-infected children and the community controls whose samples were used for determination of B-cell phenotypes. Values shown are medians (inter-quartile range) unless otherwise stated. Statistical tests used|: [a]Wilcoxon rank-sum test (Mann Whitney test), [b]Chi-squared test.

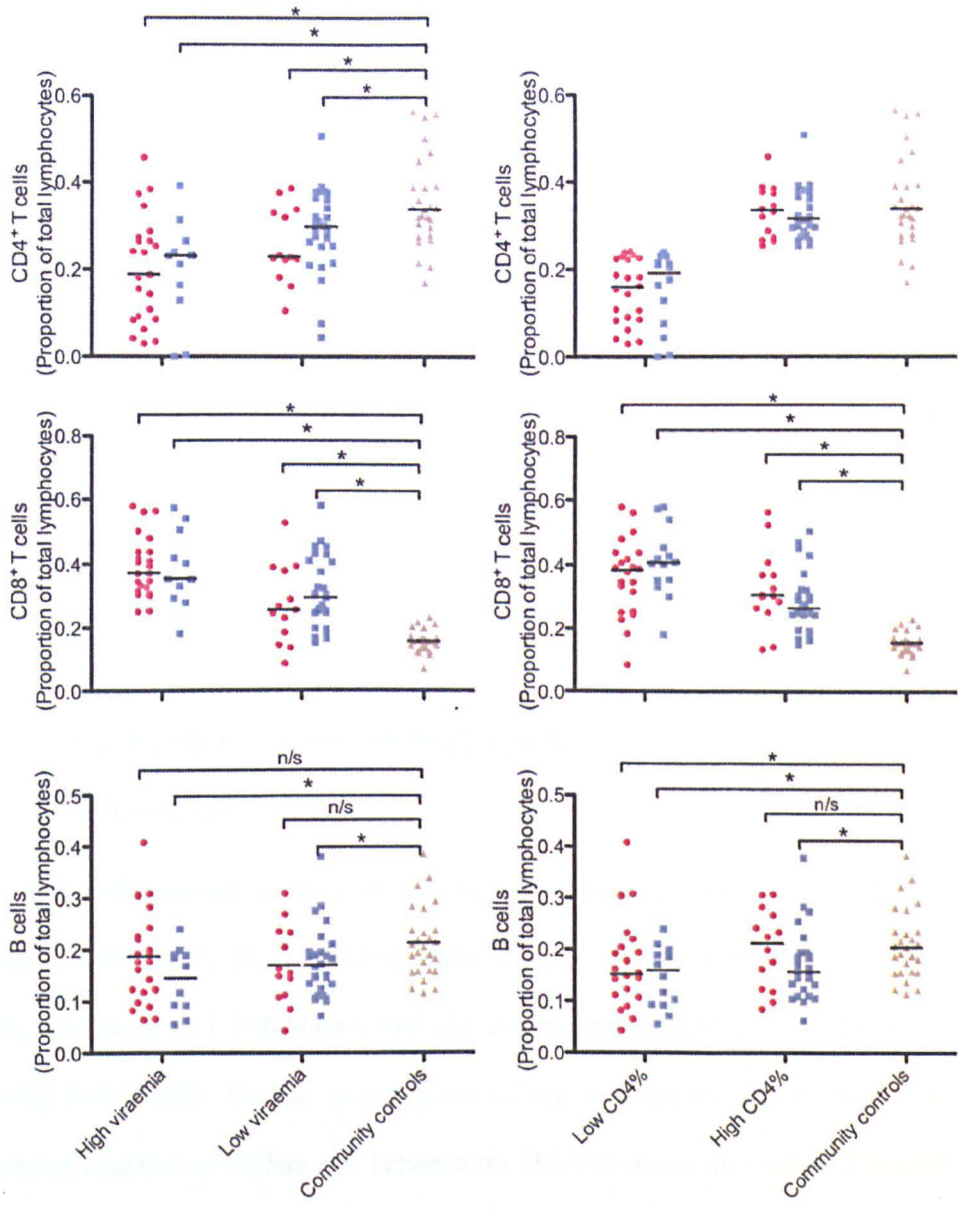

Figure 3.2: Frequencies of lymphocyte lineages in the HIV-infected and community control children after stratifying the children on the basis of their viral loads or CD4+ T-cell percentages. Blue squares represent children who were treated with HAART, red circles represent children who were HAART naïve and brown triangles represent the community controls. Horizontal line shows the median frequency for each sub-group. High viraemia group: >/=5000 RNA copies/ml, low viraemia group: < 5000 RNA copies/ml, low CD4 percentage group: <25% and high CD4 percentage group : >/=25%. Statistical test used: Wilcoxon rank-sum test. * represents $p<0.05$.

3.4.2 Comparison of different children's groups with regard to frequencies of various B-cell subsets

When the HIV-infected cohort was compared with community controls, there were no statistically significant differences in the frequencies of atypical memory B cells, activated mature B cells, plasmablasts and immature/transitional B cells. However, as previously reported, HIV-infected children had lower frequencies of resting memory B cells when compared with community controls (6.42% [IQR, 3.64%-7.67%] versus 11.25% [IQR, 8.11%-14.83%] P<0.0005) [232, 234]. In addition, frequencies of naïve B cells were lower in the HIV-infected children (47.30% [IQR, 35.88%-66.45%]) when compared with community controls (57.45% [IQR, 54.20%-63.90%] P=0.0433) [234].

3.4.3 Comparisons after stratifying the HIV-infected children on the basis of viraemia and HAART treatment

The median frequency of naïve B cells was significantly lower in the high viraemia group (37.35%[IQR, 23.7%-48.2%]) than in both the low viraemia group (62.75% [IQR, 45.4%-70.1%] P<0.00005) and the community controls (57.5% [IQR, 54.2%-63.9%] P<0.00005). Further stratification of the children on the basis of HAART treatment revealed no differences between the HAART-naïve and HAART treated sub-groups within both high viraemia and low viraemia groups. In addition, there were no differences when the HAART-naïve children and the HAART-receiving children within the low viraemia group were compared with community controls, suggesting that both control of viraemia upon initiation of HAART and spontaneous control of viraemia in absence of HAART are associated with recover and maintenance of naïve B-cell compartment, respectively. On the other hand, the two sub-groups within the high viraemia group had lower frequencies of naïve B cells when compared with community controls. Notably, the lowly viraemic children who were on HAART had higher

frequencies of naïve B cells than the two sub-groups of highly viraemic children, reinforcing the impression that successful HAART with control of viraemia is associated with recovery of the naïve B-cell compartment.

Both the high viraemia group and the low viraemia group (i.e. regardless of viraemia level) had similar median frequencies of resting memory B cells (5.2% [IQR, 3.5%-7.1%] and 6.8% [IQR, 3.6%-8.5%] respectively, P=0.1809), which were both lower when compared with the community controls (11.3% [IQR, 8.1%-14.8%] P<0.00005 in both cases). There were no differences between the HAART-naïve and HAART-treated children within both the high and low viraemia groups with regard to frequencies of resting memory B cells. Furthermore, all the HAART-naïve and HAART-treated sub-groups, regardless of viraemia, had comparable frequencies of resting memory B cells that were all low when compared with community controls, suggesting that, like in adults, the memory B-cell compartment is permanently damaged.

There was viraemia dependency on the frequency of activated mature B cells whereby the high viraemia group had significantly higher median frequency (9.0% [IQR, 7.3%-13.1%]) than both the low viraemia group (3.0% [IQR, 1.8%-6.3%] P<0.00005) and the community controls (4.0% [IQR, 2.8%-4.7%] P<0.00005). The low viraemia group was comparable with community controls in frequencies of activated mature B cells (P=0.3847), suggesting that active viraemia contributes directly to the aberrant activation of B cells. Interestingly, the high viraemia subgroup that was on HAART had lower frequencies of activated mature B cells when compared with the high viraemia subgroup that was HAART-naïve (10.1% [IQR, 8.3%-14.4%] versus 5.5% [IQR, 2.6%-8.8%] P=0.0067), implying that initiation of HAART alone could lead to restoration of normal frequencies of activated mature B cells even before the viral load drops to below 5000 RNA copies per ml. The HAART-naïve children who had low viraemia were comparable with the HAART-treated children who also had low viraemia with regard to

frequencies of activated mature B cells (5.2% [IQR, 1.9%-7.2%] versus 2.4% [IQR, 1.8%-5.7%] P=0.2705).

A similar trend was observed in atypical memory B cells whereby the high viraemia group had higher median frequency (28.6% [IQR, 20.5%-39.2%]) than the low viraemia group (14.9% [IQR, 9.7%-23.0%] P<0.00005) and the community controls (12.6% [IQR, 10.4%-16.4%] P<0.00005). There were no differences between the HAART-naïve and HAART-treated sub-groups in frequencies of atypical memory B cells within the high viraemia group (30.2% [IQR, 20.5%-38.9%] versus 25.9% [IQR, 17.6%-40.2%] P=0.5477). However, those children who were HAART naïve and had low viraemia showed higher frequencies of atypical memory B cells when compared with those who were on HAART and had low viraemia (18.5% [IQR, 11.4%-36.6%] versus 13.3% [IQR, 7.9%-19.6%] P=0.0128). Furthermore, while the low viraemia HAART-treated children were similar to community controls (P=0.9047), the low viraemia HAART naïve children had higher frequencies of atypical memory B cells when compared with community controls (P=0.0092).

The high viraemia group also had higher median frequency of plasmablasts (4.0% [IQR, 2.0%-7.0%]) than the low viraemia group (1.9% [IQR, 0.9%-3.6%] P=0.0021) and the community controls (1.9% [IQR, 1.3%-4.7%] P=0.0230). Further stratification on the basis of HAART treatment revealed no differences between HAART treated and HAART-naïve children within both low viraemia and high viraemia groups. Notably, the highly viraemic children who were HAART naïve had higher frequencies of plasmablasts (4.1% [IQR, 2.5%-7.1%]) than the lowly viraemic HAART treated children (1.7% [IQR, 0.9%-3.1%] P=0.0007) and the community controls (1.9% [IQR, 1.3%-4.7%] P=0.0114), suggesting that initiation of HAART with adequate control of viraemia leads to restoration in this compartment.

There were no differences in frequencies of immature/transitional B cells between the groups.

The resting memory B-cell compartment was further analysed to give the frequencies of its IgD^+ (unswitched) and IgD^- (switched) subsets. The picture was similar to that observed in total resting memory B cells above. Similar to previous reports, there were reduced frequencies of IgD^+ resting memory B cells in the HIV-infected cohort (2.24% [IQR, 1.32%-3.22%]) when compared with community controls (4.05% [IQR, 3.44%-5.49%] $P<0.00005$) [230, 231, 234]. However, similar to a previous report [231], but not others [230, 234], there were reduced frequencies of IgD^- resting memory B cells in HIV-infected children (3.03% [IQR, 1.62%-4.65%] versus 6.57% [IQR, 4.66%-7.93%] $P<0.00005$). Both high viraemia and low viraemia groups had lower frequencies of IgD^+ resting memory B cells (1.7% [IQR, 1.0%-2.9%] and 2.7% [IQR, 1.6%-3.6%] respectively) than the community controls (4.1% [IQR, 3.4%-5.5%] $P<0.00005$ and $P=0.0001$, respectively). Similarly, the two HIV-infected groups had lower median frequencies of switched resting memory B cells (2.4% [IQR, 1.7%-3.9%] and 3.2% [IQR, 1.4%-5.6%]) than the community controls (6.6% [IQR, 4.7%-7.9%] $P<0.0005$ in both cases). Notably, the low viraemia group showed partial recovery/preservation of the IgD^+ resting memory compartment when compared with the high viraemia group ($P=0.0380$). Stratification of the children further based on HAART treatment did not reveal any differences between HAART-naïve and HAART-treated children within both high viraemia and low viraemia groups with regard to frequencies of IgD^+ and IgD^- resting memory B cells. Notably, all HIV-infected subgroups had lower frequencies of IgD^+ and IgD^- resting memory B cells when compared with community controls.

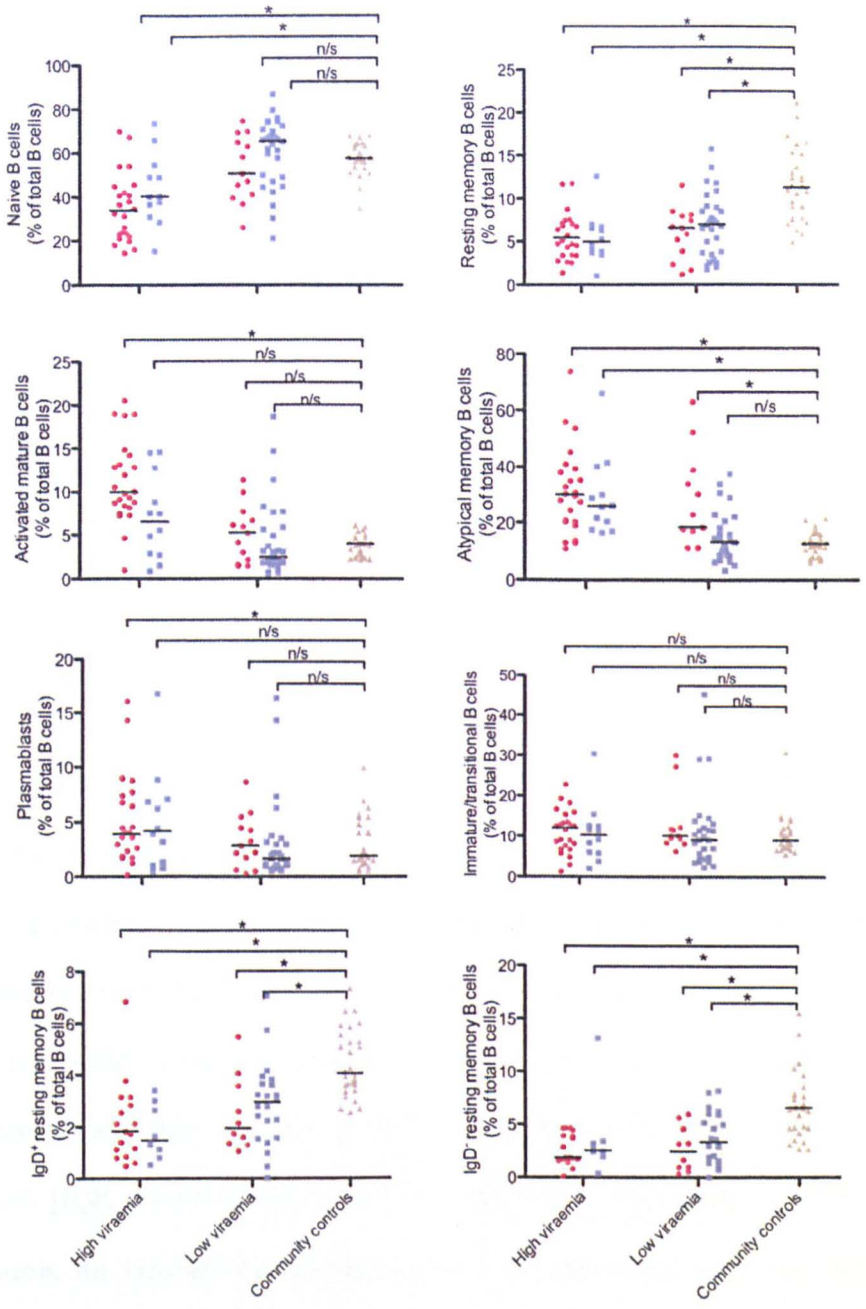

Figure 3.3: Frequencies of various B-cell subsets in the three groups of children after virological classification. Blue squares represent children who were treated with HAART, red circles represent children who were HAART naïve and brown triangles represent the community controls. Horizontal line shows the median frequency for each subgroup. High viraemia group: >/=5000 RNA copies/ml, low viraemia group: < 5000 RNA copies/ml. Statistical test used: Wilcoxon rank-sum test. * represents $p<0.05$.

3.4.4 Comparisons after stratifying the HIV-infected children on the basis of $CD4^+$ T-cell percentages and HAART treatment

Similar results with few variations were obtained when the children were classified on the basis of their immunological profile (i.e. based on the 25% $CD4^+$ T-cell percentage cut off as recommended by WHO).

Children with low $CD4^+$ T-cell percentages had lower frequencies of naïve B cells (41.0% [IQR, 24.3%-54.5%]) when compared with community controls (57.5% [IQR, 54.2%-63.9%] P=0.0003) and children with high $CD4^+$ T-cell percentages (58.0% [IQR, 39.7%-68.3%] P=0.0046). However, community controls did not differ from children having high $CD4^+$ T-cell percentages. Interestingly, there were significant differences between HAART-naïve and HAART-treated children within both high and low $CD4^+$ T-cell percentage groups. The HAART-naïve children in the low $CD4^+$ T-cell percentage group had lower frequencies of naïve B cells when compared with HAART-treated children who had low $CD4^+$ T-cell percentages (34.3% [IQR, 21.8%-48.2%] versus 49.6% [IQR, 39.4%-62.6%] P=0.0297). Similarly, the HAART naïve children in the high $CD4^+$ T-cell percentage group had lower frequencies of naïve B cells when compared with their HAART-treated counterparts (43.6% [IQR, 35.4%-62.0%] versus 66.0% [IQR, 44.8%-73.1%] P=0.0227). Moreover, when compared with community controls, the HAART-treated high $CD4^+$ T-cell percentage subgroup had comparable frequencies of naïve B cells (P=0.1608) while the HAART-naïve children with high $CD4^+$ T-cell percentages had significantly lower frequencies of naïve B cells (P=0.0218). The same trend was observed when the low $CD4^+$ T-cell percentage subgroups were compared with community controls. The HAART naïve children with low $CD4^+$ T-cell percentages also had lower frequencies of naïve B cells when compared to community controls (P=0.0001). However, their HAART treated counterparts did not differ from community controls (P=0.0927).

Similar to previous reports, both groups of children with low and high $CD4^+$ T-cell percentages had lower frequencies of resting memory B cells (4.8% [IQR, 3.4%-7.0%] and 6.9% [IQR, 4.3%-8.6%] respectively) than community controls (11.3% [IQR, 8.1%-14.8%] $P<0.0005$ in both cases). Interestingly, children with high $CD4^+$ T-cell percentages had higher frequencies of resting memory B cells when compared with the group that had low $CD4^+$ T-cell percentages ($P=0.0124$), suggesting that they had experienced partial recovery or partial preservation of this compartment. Further stratification of the children on the basis of HAART treatment revealed no differences between the HAART-naïve and HAART-treated children within both high and low $CD4^+$ T-cell percentage groups. Notably, HAART-naïve children with low $CD4^+$ T-cell percentages had significantly lower frequencies of resting memory B cells when compared with HAART-treated children who had high $CD4^+$ T-cell percentages, reinforcing the possibility of partial recovery of this compartment when $CD4^+$ T-cell frequencies recover upon initiation of HAART.

In addition, the children with low $CD4^+$ T-cell percentages had higher frequencies of activated mature B cells (7.3% [IQR, 2.7%-11.2%]) than the community controls (4.0% [IQR, 2.8%-4.7%] $P=0.0056$). Further stratification into HAART-naïve and HAART-treated children revealed significant differences between the two subgroups within both low and high $CD4^+$ T-cell percentage groups. For instance, the HAART-naïve children who had low $CD4^+$ T-cell percentages had significantly higher frequencies of activated mature B cells when compared with the HAART-treated children in the same low $CD4^+$ T-cell percentage group (9.6% [IQR, 6.1%-13.3%] versus 3.2% [IQR, 1.9%-7.5%] $P=0.0045$). Similarly, HAART-naïve children with high $CD4^+$ T-cell percentages had higher frequencies of activated mature B cells when compared with their HAART-

treated counterparts who had high CD4+ T-cell percentages (8.3% [IQR, 4.8%-12.2%] versus 2.9% [IQR, 1.8%-7.8%] P=0.0172), suggesting that HAART greatly affected the frequencies of this activated subset of B cells.

Frequencies of atypical memory B cells were elevated in the group that had low $CD4^+$ T-cell percentages (29.8% [IQR, 17.7%-40.0%]) when compared with the group that had high $CD4^+$ T-cell percentages (16% [IQR, 10.8%-24.2%] P=0.0002) and community controls (12.6% [IQR, 10.4%-16.4%] P<0.00005). Similar to observations above, further stratification of the children revealed that those who had low $CD4^+$ T-cell percentages and were HAART-naïve had higher frequencies of atypical memory B cells when compared with HAART-treated children with low $CD4^+$ T-cell percentages (35.3% [IQR, 22.0%-47.1%] versus 21.4% [IQR, 13.9%-31.2%] P=0.0231). The same was observed within the high $CD4^+$ T-cell percentage group whereby HAART naïve children had higher frequencies of atypical memory B cells when compared with HAART treated children (20.5% [IQR, 11.8%-29.4%] versus 14.9% [IQR, 8.4%-21.1%] P=0.0433), suggesting that HAART treatment had some impact on the frequencies of atypical memory B cells regardless of immunological profile.

Of note, there were no differences in frequencies of plasmablasts among the various groups of children after their stratification on the basis of $CD4^+$ T-cell percentages. However, further stratification of the children revealed that HAART-naïve children who had high $CD4^+$ T-cell percentages also had higher frequencies of plasmablasts when compared to HAART-treated children who had high $CD4^+$ T-cell percentages (3.8% [IQR, 2.3%-6.4%] versus 1.8% [IQR, 0.9%-3.3%] P=0.0259). There were no differences between the HAART-naïve and HAART-treated children in the low $CD4^+$ T-cell percentage group with regard to frequencies of plasmablasts.

Similarly, there were no differences in frequencies of immature/transitional B cells among the various groups of children after their stratification on the basis of $CD4^+$ T-cell percentages. However, further stratification of the children revealed that HAART-naïve children who had high $CD4^+$ T-cell percentages also had higher frequencies of immature/transitional B cells when compared with HAART-treated children who had high $CD4^+$ T-cell percentages (11.6% [IQR, 9.1%-15.0%] versus 7.1% [IQR, 4.5%-11.9%] P=0.0115). There were no differences between the HAART-naïve and HAART-treated children in the low $CD4^+$ T-cell percentage groups with regard to frequencies of immature/transitional B cells.

Further analysis of the resting memory B-cell compartment revealed low frequencies of IgD^+ resting memory B cells in low $CD4^+$ T-cell percentage group (1.7% [IQR, 1.1%-3.0%]) and high $CD4^+$ T-cell percentage group (2.8% [IQR, 1.6%-3.6%]) when compared with community controls (4.1% [IQR, 3.4%-5.5%] P<0.00005 and P=0.0001 respectively). There was also a significant but partial recovery/preservation of IgD^+ resting memory B cells in the group that had high $CD4^+$ T-cell percentages when compared with the group that had low $CD4^+$ T-cell percentages (P=0.0344). Similarly, IgD^- resting memory B cells were in low frequencies in both low and high $CD4^+$ T-cell percentage groups (2.6% [IQR, 1.1%-3.5%] and 3.6% [IQR, 1.7%-5.2%] respectively) when compared with community controls (6.6% [4.7%-7.9%] P<0.00005 and P=0.0001 respectively). However, there was only a statistically non-significant trend towards partial recovery/preservation of this compartment in the high $CD4^+$ T-cell percentage group (P=0.0702). Upon further stratification of the children on the basis of their HAART treatment status, there were no differences between the HAART-naïve and HAART-treated children with regard to IgD^- or IgD^+ resting memory B cells within both low and high $CD4^+$ T-cell percentage groups.

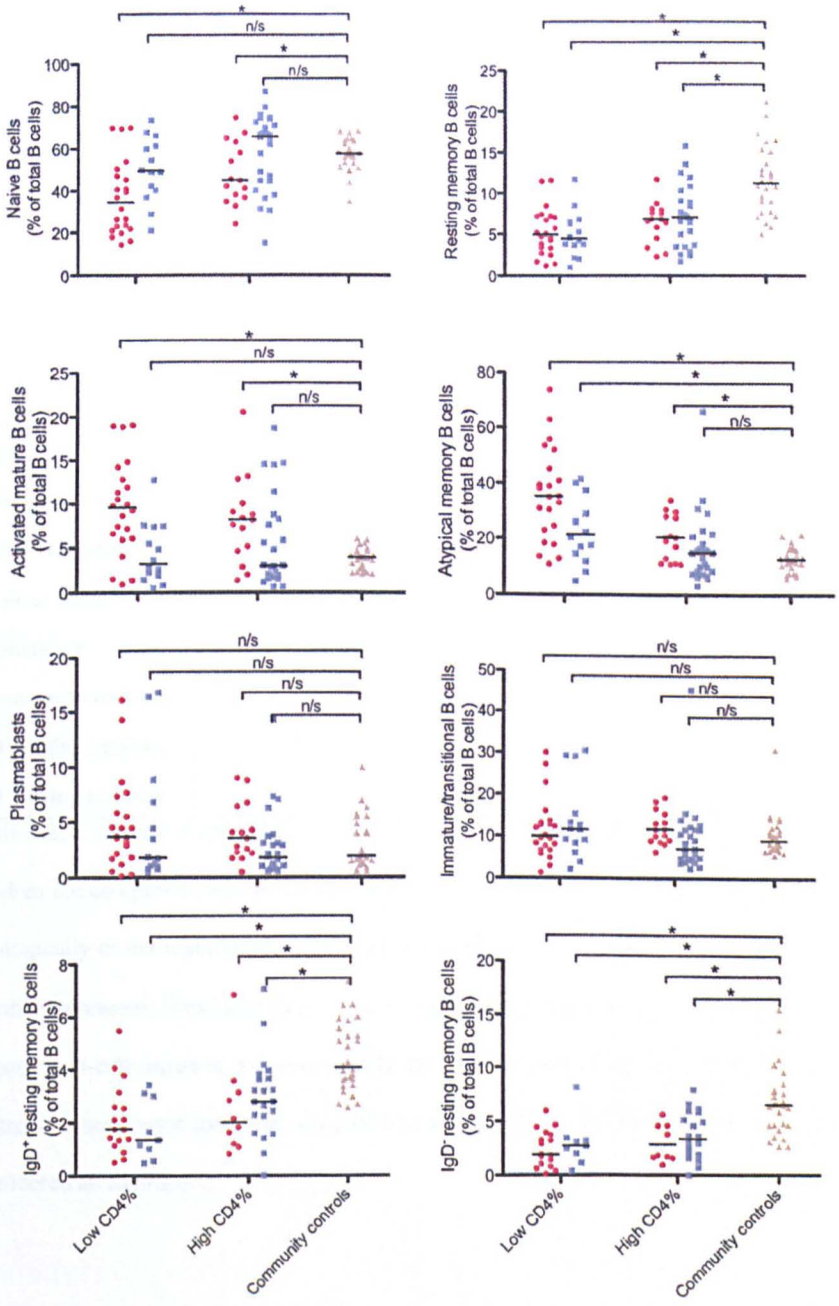

Figure 3.4: Frequencies of various B-cell subsets in the three groups of children after immunological classification. Blue squares represent children who were treated with HAART, red circles represent children who were HAART naïve and brown triangles represent the community controls. Horizontal line shows the median frequency for each sub-group. Low CD4 percentage group: <25% and high CD4 percentage group : >/=25%. Statistical test used: Wilcoxon rank-sum test. * represents $p<0.05$.

3.4.5 Summary

	Virological classification				Immunological classification			
	High viraemia		Low viraemia		Low $CD4^+$ T-cell %		High $CD4^+$ T-cell %	
	No HAART	HAART	No HAART	HAART	No HAART	HAART	No HAART	HAART
Naïve	⇓	⇓	⇔	⇔	⇓	⇔	⇓	⇔
Resting memory	⇓	⇓	⇓	⇓	⇓	⇓	⇓	⇓
Activated mature	⇑	⇔	⇔	⇔	⇑	⇔	⇑	⇔
Atypical memory	⇑	⇑	⇑	⇔	⇑	⇑	⇑	⇔
Plasmablast	⇑	⇔	⇔	⇔	⇔	⇔	⇑	⇔
Immature/transitional	⇔	⇔	⇔	⇔	⇔	⇔	⇔	⇔
IgD^+ resting memory	⇓	⇓	⇓	⇓	⇓	⇓	⇓	⇓
IgD^- resting memory	⇓	⇓	⇓	⇓	⇓	⇓	⇓	⇓

Table 3.2: Summary of the effect of HIV on the various subsets of B cells when the HIV-infected children are compared with community control children. The HIV-infected children can be stratified virologically or immunologically with further stratification on the basis of their HAART treatment status. Symbols represent: ⇓-reduced frequency, ⇔-similar frequency and ⇑-increased frequency of the respective B-cell subset in the group of HIV-infected children when they are compared with community controls. Groups were compares using Wilcoxon rank-sum test (Mann-Whitney test) with $p<0.005$ considered as significant.

When compared with community controls, all HIV-infected subgroups of children, regardless of mode of classification, had decreased frequencies of total resting memory B cells, unswitched resting memory B cells and switched memory B cells. They also all had unaffected frequencies of immature/transitional B cells. Low frequencies of naïve B cells were observed in all high viraemia groups upon virological classification and in

HAART naïve groups upon immunological classification, suggesting that viral load and absence of HAART, and not $CD4^+$ T-cell depletion, were important in determining the frequencies of naïve B cells. Elevated frequencies of activated mature B cells were observed in HAART naïve highly viraemic children upon virological classification and in all HAART naïve groups upon immunological classification, suggesting that viral load and absence of HAART were the major determinants of frequencies of this subset. Elevated frequencies of plasmablasts in HAART naïve highly viraemic children and HAART naïve children with high $CD4^+$ T-cell percentages also suggest that viral load and HAART treatment are important determinants of frequencies of plasmablasts. Atypical memory B cells were increased in all subgroups upon both virological and immunological classification with the exception of the HAART-treated low viraemia group and the HAART treated high $CD4^+$ T-cell percentage group, suggesting some dependence on disease progression.

3.4.6 Statistical interaction (effect modification) between various HIV states and age in the acquisition of resting memory B cells

To test for interaction between age, treatment with HAART and high viraemia or low $CD4^+$ T-cell percentage with regard to the age-related changes in frequencies of resting memory B-cell subsets, a predictive linear regression model was used with the predicted frequency of resting memory B cells as the dependent variable and age and level of viraemia or level of $CD4^+$ T-cell percentage with/without HAART as independent variables. Adjustment was done for gender, hemoglobin content and total lymphocyte counts. The F statistic for the interaction term and significance level were reported [235]. The F statistic was an indication of the strength of effect modification by viraemia and treatment. It is a function of the differences in the regression lines of the different groups of children.

3.4.7 Statistical interaction after stratifying the children on the basis of viraemia and HAART

When the various groups were compared with regard to accumulation of resting memory B cells, notable trends were observed. As would be expected, the community controls showed a gradual increase in predicted frequencies of resting memory B cells with increase in age, probably due to exposure to environmental antigens and pathogens with advancement in age. Similarly, the low viraemia groups, with and without HAART, showed parallel increments of predicted frequencies of resting memory B cells with age, though they remained consistently lower than the community controls. However, the high viraemia groups did not show a similar increase with age but instead showed an age-dependent (hence HIV-exposure dependent) decrease of total resting memory B cells. The interaction was statistically significant when the high viraemia HAART-naïve group was compared with community controls. Significant interactions were also observed when the high viraemia HAART-naïve group was compared with the low viraemia groups, suggesting that controlling viraemia could enable HIV-infected children to accumulate resting memory B cells as they advance in age.

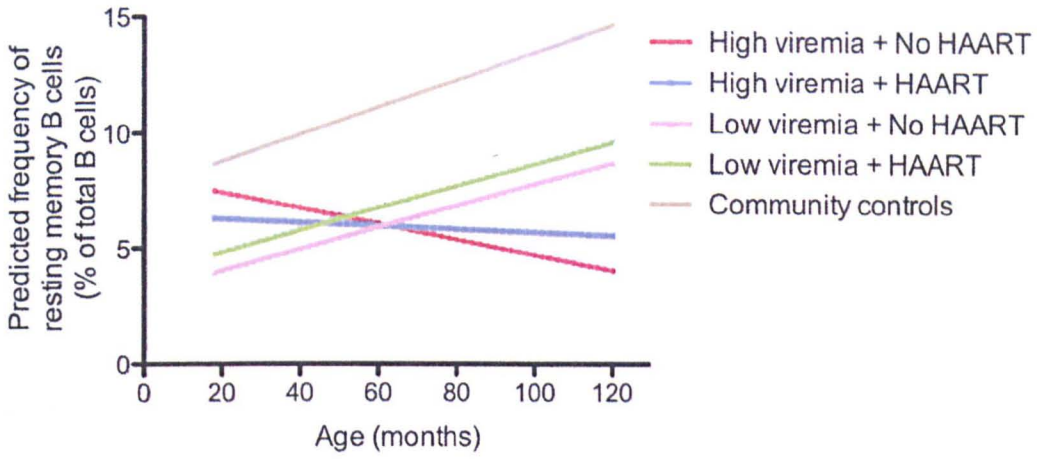

Figure 3.5: Predicted frequencies of resting memory B-cell compartment after stratifying the HIV-infected children on the basis of their level of viraemia and HAART treatment status. The Predxcon STATA package was used to make linear regression predictions that were then plotted.

F statistic	High viraemia + No HAART	High viraemia + HAART	Low viraemia + No HAART	Low viraemia + HAART	Community Controls
High viraemia +No HAART		F(1,25)=1.09	F(1,23)=5.10	F(1,41)=6.08	F(1,41)=6.52
High viraemia +HAART			F(1,13)=1.24	F(1,31)=0.52	F(1,31)=3.09
Low viraemia +No HAART				F(1,29)=0.11	F(1,29)=0.28
Low viraemia +HAART					F(1,47)=0.05
Community Controls					

Table 3.3: The F statistics for the interaction of age*(viraemia with/without HAART) with regard to accumulation of resting memory B cells. Children were stratified on the basis of viraemia and HAART treatment. Pairwise comparisons were done between the various groups using the Predixcon STATA package for linear regression prediction. Significant results are in red text.

P value	High viraemia + No HAART	High viraemia + HAART	Low viraemia + No HAART	Low viraemia + HAART	Community Controls
High viraemia +No HAART		0.3068	0.0338	0.0179	0.0145
High viraemia +HAART			0.2862	0.4777	0.0888
Low viraemia +No HAART				0.7415	0.5987
Low viraemia +HAART					0.8219
Community Controls					

Table 3.4: The P values for the interaction of age*(viraemia with/without HAART) with regard to the accumulation of resting memory B cells. Children were stratified on the basis of viraemia and HAART treatment. Pairwise comparisons were done between the various groups using the predixcon stata package for linear regression prediction. P values less than 0.05 were considered as significant for interaction. Significant results are in red text.

Statistical interaction was also observed in IgD^- (switched) resting memory B cells whereby both community controls and low viraemia groups showed a gain with age while the high viraemia groups showed no age-related gain of predicted frequencies. Statistically significant interactions were observed when the HAART-naïve high viraemia group was compared with community controls, low viraemia HAART naïve group and low viraemia HAART-treated group. Similar significant interaction was observed when high viraemia HAART-treated children were compared with low viraemia HAART-naïve children, suggesting that viraemia and not HAART could be the main determinant on whether these children gained frequencies of IgD^- resting memory B cells.

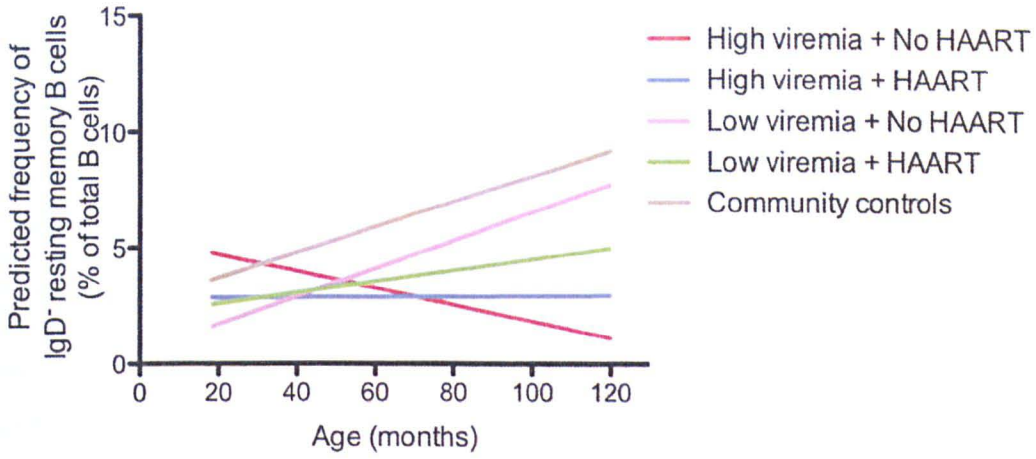

Figure 3.6: Predicted frequencies of IgD$^-$ resting memory B-cell compartment after stratifying the HIV-infected children on the basis of their level of viraemia and HAART treatment status.

The predxcon stata package was used to make linear regression predictions that were then plotted.

F statistic	High viraemia + No HAART	High viraemia + HAART	Low viraemia + No HAART	Low viraemia + HAART	Community Controls
High viraemia +No HAART		F(1,16)=5.90	F(1,15)=21.43	F(1,30)=5.84	F(1,36)=10.6
High viraemia +HAART			F(1,6)=9.86	F(1,21)=0.01	F(1,27)=3.14
Low viraemia +No HAART				F(1,20)=4.75	F(1,26)=0.30
Low viraemia +HAART					F(1,41)=1.52
Community Controls					

Table 3.5: The F statistics for the interaction of age*(viraemia with/without HAART) with regard to accumulation of IgD$^-$ resting memory B cells. Children were stratified on the basis of viraemia and HAART treatment. Pairwise comparisons were done between the various groups using the predixcon stata package for linear regression prediction. Significant results are in red text.

P value	High viraemia + No HAART	High viraemia + HAART	Low viraemia + No HAART	Low viraemia + HAART	Community Controls
High viraemia +No HAART		0.0273	0.0003	0.0220	0.0025
High viraemia +HAART			0.0201	0.9198	0.0876
Low viraemia +No HAART				0.0414	0.5883
Low viraemia +HAART					0.2247
Community Controls					

Table 3.6: The P values for the interaction of age*(viraemia with/without HAART) with regard to the accumulation of IgD^- resting memory B cells. Children were stratified on the basis of viraemia and HAART treatment. Pairwise comparisons were done between the various groups using the predixcon stata package for linear regression prediction. P values less than 0.05 were considered as significant for interaction. Significant results are in red text.

There was no interaction between age and level of viraemia with regard to the acquisition of IgD^+ (unswitched) resting memory B cells. Further stratification of the HIV-infected children on the basis of HAART treatment did not reveal any interaction between the various sub-groups.

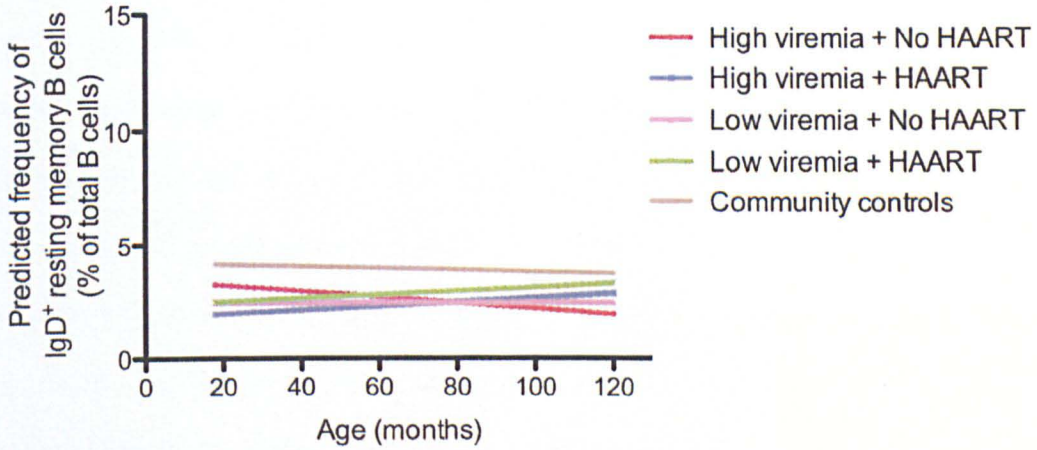

Figure 3.7: Predicted frequencies of IgD^+ resting memory B cells after stratifying the HIV-infected children on the basis of their level of viraemia and HAART treatment status.The predxcon stata package was used to make linear regression predictions that were then plotted.

F statistic	High viraemia + No HAART	High viraemia + HAART	Low viraemia + No HAART	Low viraemia + HAART	Community Controls
High viraemia +No HAART		F(1,16)=0.35	F(1,15)=0.44	F(1,30)=1.00	F(1,36)=0.13
High viraemia +HAART			F(1,6)=0.01	F(1,21)=0.01	F(1,27)=0.09
Low viraemia +No HAART				F(1,20)=0.17	F(1,26)=0.02
Low viraemia +HAART					F(1,41)=1.18
Community Controls					

Table 3.7: The F statistics for the interaction of age*(viraemia with/without HAART) with regard to the accumulation of IgD+ resting memory B cells. Children were stratified on the basis of viraemia and HAART treatment. Pairwise comparisons were done between the various groups using the predixcon stata package for linear regression prediction. Significant results are in red text.

P value	High viraemia + No HAART	High viraemia + HAART	Low viraemia + No HAART	Low viraemia + HAART	Community Controls
High viraemia +No HAART		0.5635	0.5195	0.3264	0.7207
High viraemia +HAART			0.9100	0.9067	0.7667
Low viraemia +No HAART				0.6839	0.9026
Low viraemia +HAART					0.2843
Community Controls					

Table 3.8: The P values for the interaction of age*(viraemia with/without HAART) with regard to the accumulation of IgD+ resting memory B cells. Children were stratified on the basis of viraemia and HAART treatment. Pairwise comparisons were done between the various groups using the predixcon stata package for linear regression prediction. P values less than 0.05 were considered as significant for interaction. There were no significant differences between the groups of children.

3.4.8 Statistical interaction after stratifying the children on the basis of $CD4^+$ T-cell percentages and HAART

Similar trends were observed between predicted frequencies of resting memory B cells, age and $CD4^+$ T-cell percentage with/without HAART when the children were classified based on their immunological profile and HAART treatment status. Similar to community controls, the high $CD4^+$ T-cell percentages groups showed an increase of predicted frequencies of resting memory B cells with age, though consistently lower than the community controls. The low $CD4^+$ T-cell groups did not show a similar increase with age but instead showed an age-dependent (hence exposure-dependent) decrease of total resting memory B cells. The interaction was statistically significant when HAART-naïve children with low $CD4^+$ T-cell percentages were compared with community controls with regard to frequencies of resting memory B cells. All other pair-wise comparisons were not statistically significant.

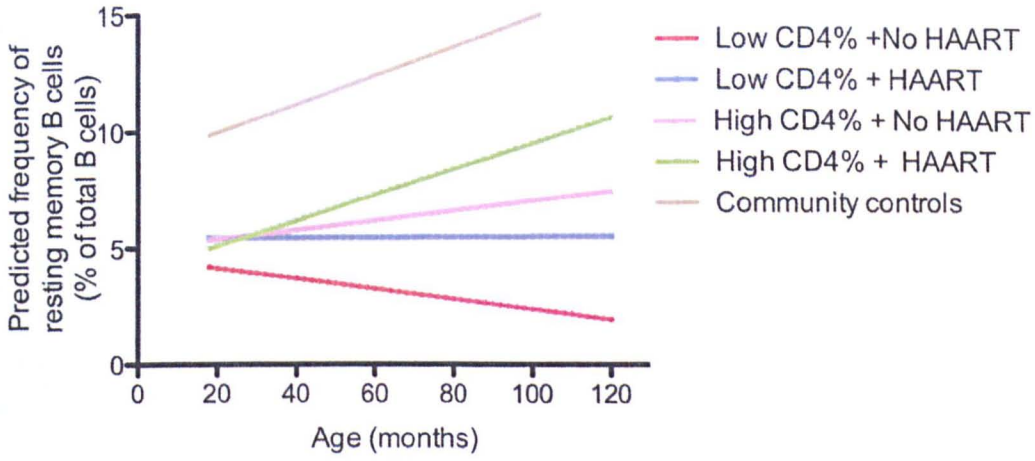

Figure 3.8: Predicted frequencies of resting memory B cells after stratifying the HIV-infected children on the basis of their CD4+ T-cell percentages with/without HAART. The predxcon stata package was used to make linear regression predictions that were then plotted.

F statistic	Low CD4 + No HAART	Low CD4 + HAART	High CD4 + No HAART	High CD4 + HAART	Community Controls
Low CD4 +No HAART		F(1,23)=0.29	F(1,23)=1.22	F(1,33)=3.64	F(1,36)=6.02
Low CD4 +HAART			F(1.21)=0.23	F(1,31)=0.50	F(1,34)=1.52
High CD4 +No HAART				F(1,31)=0.27	F(1,34)=0.63
High CD4 +HAART					F(1,44)=0.01
Community Controls					

Table 3.9: The F statistics for the interaction of age*(CD4% with/without HAART) with regard to accumulation of resting memory B cells. Children were stratified on the basis of CD4+ T-cell percentages and HAART treatment. Pairwise comparisons were done between the various groups using the predixcon stata package for linear regression prediction. Significant results are in red text.

P value	Low CD4 + No HAART	Low CD4 + HAART	High CD4 + No HAART	High CD4 + HAART	Community Controls
Low CD4 +No HAART		0.5934	0.2802	0.0650	0.0192
Low CD4 +HAART			0.6336	0.4840	0.2266
High CD4 +No HAART				0.6102	0.4331
High CD4 +HAART					0.9388
Community Controls					

Table 3.10: The P values for the interaction of age*(CD4% with/without HAART) with regard to the accumulation of resting memory B cells. Children were stratified on the basis of their CD4+ T-cell percentages and HAART treatment. Pairwise comparisons were done between the various groups using the predixcon stata package for linear regression prediction. P values less than 0.05 were considered as significant for interaction. Significant results are in red text.

A similar trend was observed in IgD^- (switched) resting memory B cells whereby both community controls and high $CD4^+$ T-cell percentage groups showed a gain of predicted frequencies. Interestingly, the HAART-treated group with low $CD4^+$ T-cell percentages did not show any age-dependent (exposure dependent) reduction in predicted frequencies of IgD^- resting memory B cells. However, the HAART-naive group with low $CD4^+$ T-cell percentages showed an age-dependent (exposure dependent) reduction in predicted frequencies of IgD^- resting memory B cells. The interaction was statistically significant when the HAART-naïve group with low $CD4^+$ T-cell percentages was compared with community controls.

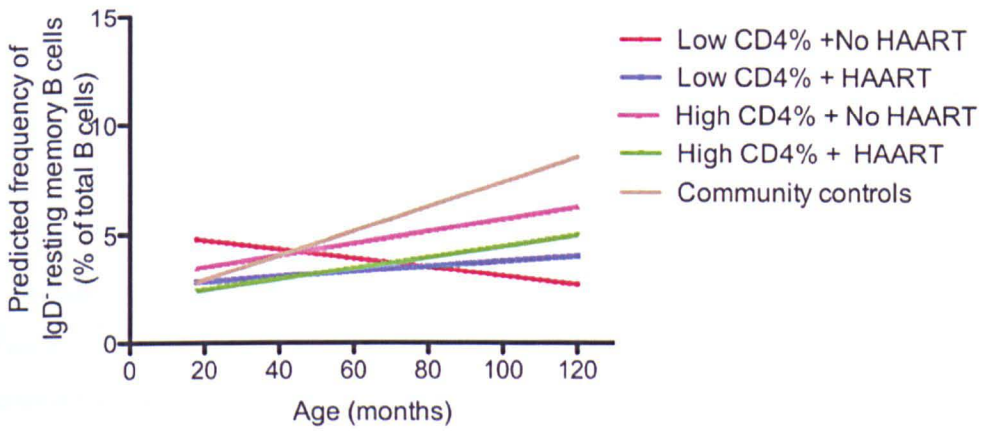

Figure 3.9: Predicted frequencies of IgD- resting memory B cells after stratifying the HIV-infected children on the basis of their $CD4^+$ T-cell percentages and HAART treatment status. The predxcon stata package was used to make linear regression predictions that were then plotted.

F statistic	Low CD4 + No HAART	Low CD4 + HAART	High CD4 + No HAART	High CD4 + HAART	Community Controls
Low CD4 +No HAART		F(1,14)=2.32	F(1,15)=2.36	F(1,24)=3.75	F(1,32)=9.11
Low CD4 +HAART			F(1,12)=0.20	F(1.21)=0.17	F(1,29)=1.62
High CD4 +No HAART				F(1,22)=0.67	F(1,30)=1.43
High CD4 +HAART					F(1,39)=1.34
Community Controls					

Table 3.11: The F statistics for the interaction of age*(CD4% with/without HAART) with regard to accumulation of IgD- resting memory B cells. Children were stratified on the basis of CD4+ T-cell percentages and HAART treatment. Pairwise comparisons were done between the various groups using the predixcon stata package for linear regression prediction. Significant results are in red text.

P value	Low CD4 + No HAART	Low CD4 + HAART	High CD4 + No HAART	High CD4 + HAART	Community Controls
Low CD4 +No HAART		0.1497	0.1451	0.0647	0.0050
Low CD4 +HAART			0.6599	0.6812	0.2133
High CD4 +No HAART				0.4208	0.2415
High CD4 +HAART					0.2541
Community Controls					

Table 3.12: The P values for the interaction of age*(CD4% with/without HAART) with regard to the accumulation of IgD- resting memory B cells. Children were stratified on the basis of their $CD4^+$ T-cell percentages and HAART treatment. Pairwise comparisons were done between the various groups using the predixcon stata package for linear regression prediction. P values less than 0.05 were considered as significant for interaction. Significant results are in red text.

Similar to observations after virological stratification, there was no interaction with regard to IgD^+ resting memory B cells when the children were stratified based on their immunological profile and HAART treatment.

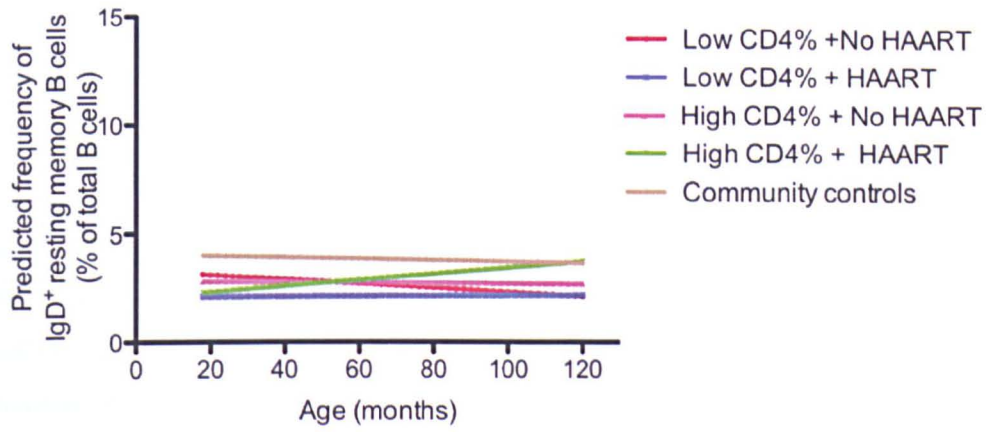

Figure 3.10: Predicted frequencies of IgD+ resting memory B cells after stratifying the HIV-infected children on the basis of their CD4+ T-cell percentages and HAART treatment status.The predxcon stata package was used to make linear regression predictions that were then plotted.

F statistic	Low CD4 + No HAART	Low CD4 + HAART	High CD4 + No HAART	High CD4 + HAART	Community Controls
Low CD4 +No HAART		F(1,14)=0.22	F(1,15)=0.04	F(1,24)=2.65	F(1,32)=0.35
Low CD4 +HAART			F(1,12)=0.01	F(1,21)=0.40	F(1,29)=0.04
High CD4 +No HAART				F(1,22)=0.00	F(1,30)=0.02
High CD4 +HAART					F(1,39)=2.44
Community Controls					

Table 3.13: The F statistics for the interaction of age*(CD4% with/without HAART) with regard to the accumulation of IgD+ resting memory B cells. Children were stratified on the basis of $CD4^+$ T-cell percentages and HAART treatment. Pairwise comparisons were done between the various groups using the predixcon stata package for linear regression prediction. There were no significant differences between the groups of children.

P value	Low CD4 + No HAART	Low CD4 + HAART	High CD4 + No HAART	High CD4 + HAART	Community Controls
Low CD4 +No HAART		0.6468	0.8432	0.1169	0.5610
Low CD4 +HAART			0.9332	0.5363	0.8480
High CD4 +No HAART				0.9446	0.8951
High CD4 +HAART					0.1267
Community Controls					

Table 3.14: The P values for the interaction of age*(CD4% with/without HAART) with regard to the accumulation of IgD+ resting memory B cells. Children were stratified on the basis of their $CD4^+$ T-cell percentages and HAART treatment. Pairwise comparisons were done between the various groups using the predixcon stata package for linear regression prediction. P values less than 0.05 were considered as significant for interaction. There were no significant differences between the groups of children.

3.4.9 Correlation of B-cell subsets with population characteristics among the HIV-infected children

Since B-cell defects could be as a result of either $CD4^+$ T-cell depletion, direct effect of viral factors or advancement in age, correlations between frequencies of various B-cell subsets and viral load, $CD4^+$ T-cell percentages, treatment with HAART and age among the HIV-infected children were checked.

	Naïve	Resting memory	Activated mature	Atypical memory	Plasmablasts	Immature/ Transitional	IgD^+ Resting memory	IgD^- Resting memory
Age(Months)	0.0875 (1.00)	0.0986 (1.00)	-0.1676 (1.00)	-0.1054 (1.00)	-0.2026 (1.00)	-0.1454 (1.00)	0.0842 (1.00)	0.2041 (1.00)
Viral load	-0.5856 (<0.05)	-0.1952 (1.00)	0.5488 (<0.05)	0.5265 (<0.05)	0.3061 (1.00)	0.1423 (1.00)	-0.3326 (0.48)	-0.2577 (1.00)
CD4+ T-cell %	0.4071 (<0.05)	0.3514 (0.09)	-0.2057 (1.0000)	-0.4413 (<0.05)	-0.1674 (1.00)	-0.181 (1.00)	0.3499 (0.33)	0.3079 (0.80)
HAART	0.3896 (<0.05)	0.0958 (1.00)	-0.4173 (<0.05)	-0.413 (<0.05)	-0.2264 (1.00)	-0.1583 (1.00)	0.1437 (1.00)	0.1809 (1.00)

Table 3.15: Spearman's rho (P value) for correlation between the frequencies of various B-cell subsets and the population characteristics (age, viral load, $CD4^+$ T-cell percentages and HAART treatment). Spearman correlation with Bonferroni adjustment were used. Adjusted P values below 0.05 were considered as significant. Significant results are in red text.

Frequencies of naïve B cells correlated inversely with viral load, directly with $CD4^+$ T-cell percentages and directly with HAART treatment, suggesting that viraemic states, low $CD4^+$ T-cell percentages and untreated state are associated with reduced frequencies of naïve B cells. There was no significant correlation between resting memory B cells and any of the considered population characteristics. However, there was a trend towards a direct correlation between resting memory B cells and $CD4^+$ T-cell percentages (rho=0.35, P=0.09), implying that recovery/maintenance of $CD4^+$ T cells could be associated with accumulation/maintenance of resting memory B cells. Frequencies of activated mature B cells correlated directly with viral load, inversely with $CD4^+$ T-cell percentages and inversely with HAART treatment, suggesting that controlling viraemia with HAART and subsequent recovery of $CD4^+$ T-cell percentages could be important in the contraction of this compartment. Interestingly, the correlation trends observed with atypical memory B cells were a mirror image of those observed with naïve B cells, suggesting that the reduction of frequencies of naïve B cells in viraemic, untreated or $CD4^+$ T-cell lymphopenic states could be as a result of expansion of the atypical memory B cells compartment. Namely, the frequencies of atypical memory B cells correlated directly with viral load, inversely with $CD4^+$ T-cell percentages and inversely with HAART treatment. While the exact origin of atypical memory B cells remains unknown, the naïve B-cell compartment remains as the most likely source. Atypical memory B cells have been shown to have immunoglobulin gene somatic hyper mutations and replication histories that are higher than those of naïve B cells but lower than those of classical memory B cells, making classical memory B cells an unlikely source and naïve B cells a likely source. However, the existence of low frequencies of atypical memory B cells in healthy individuals raises the possibility of them being a unique lineage that just expands upon chronic exposure to high HIV viraemia [227].

No significant correlations were observed within other B-cell subsets.

Notably, the correlation results should be interpreted with caution since viral load, $CD4^+$ T-cell percentages and HAART treatment are not entirely independent variables. Treatment with HAART could control viraemia which in turn could allow the $CD4^+$ T-cell percentages to recover. Indeed, the three variables showed some degree of multicollinearity.

	HIV status	Viral load	$CD4^+$ T-cell %	HAART
HIV status	1			
Viral load	-0.5988 (<0.00005)	1		
$CD4^+$ T-cell %	0.3929 (<0.00005)	-0.5152 (<0.00005)	1	
HAART	0.8163 (<0.00005)	-0.6658 (<0.00005)	0.4326 (<0.00005)	1

Table 3.16: Multicollinearity between HIV-infection, viral loads, CD4+ T-cell percentages and HAART treatment. Spearman's rho(p values) for correlations between the various population characteristics. Spearman's correlation with Bonferroni adjustments were done. P values below 0.05 were considered as significant. Significant results are in red text.

3.4.10 Correlation of the various B-cell subsets with other B cell subsets

Since a defect in one B-cell subset could be related to defects in other B-cell subsets, probably due to being driven by the same factors, correlations were checked amongst the various B-cell subsets. Interestingly, frequencies of naïve B cells correlated inversely with frequencies of activated phenotypes namely activated mature B cells, atypical memory B cells and plasmablasts. The frequencies of activated mature B cells correlated directly with frequencies of atypical memory B cells and plasmablasts.

However, frequencies of atypical memory B cells did not correlate with frequencies of plasmablasts. The frequencies of resting memory B cells correlated directly with the frequencies of its subsets i.e. IgD^- and IgD^+ resting memory B cells. Similarly, the frequencies of IgD^- resting memory B cells correlated directly with the frequencies if IgD^+ resting memory B cells, suggesting that the same factors probably affected them in the same way in HIV patients.

	Naive	Resting memory	Activated mature	Atypical memory	Plasmablasts	Immature/ transitional	IgD^+ Resting memory	IgD^- Resting memory
Naive	1							
Resting memory	0.1006 (1.0000)	1						
Activated mature	-0.8248 (<0.00005)	0.0462 (1.0000)	1					
Atypical memory	-0.8267 (<0.00005)	-0.3088 0.1129	0.6294 (<0.00005)	1				
Plasmablasts	-0.4949 (<0.00005)	0.0826 (1.0000)	0.5456 (<0.00005)	0.2453 0.6626	1			
Immature/ transitional	-0.1114 (1.0000)	-0.323 0.0718	-0.0216 (1.0000)	-0.1097 (1.0000)	-0.0259 (1.0000)	1		
IgD^+ resting memory	0.1201 (1.0000)	0.7685 (<0.00005)	-0.0171 (1.0000)	-0.2948 0.1724	-0.0094 (1.0000)	-0.1143 (1.0000)	1	
IgD^- resting memory	0.0096 (1.0000)	0.8508 (<0.00005)	0.0905 (1.0000)	-0.2146 (1.0000)	0.1052 (1.0000)	-0.1915 (1.0000)	0.6826 (<0.00005)	1

Table 3.17: Spearman's rho (P values) for correlations between various B-cell subsets. Spearman's correlation with Bonferroni adjustments were done. P values below 0.05 were considered as significant. Significant results are in red text.

3.4.11 Multivariate analysis of effect of various population characteristics on B-cell subset distribution with adjustment for age

The distribution of lymphocytes subsets in children can vary with age. As a result, immunological studies of children try to age-match the different groups of children. In this study, though an effort was done to match the different treatment groups of children by age, subsequent stratification of the children on the basis of viral loads and $CD4^+$ T-cell percentages gave groups that were not perfectly matched. Furthermore, the community controls were slightly older than the HIV-infected children, though the differences in age were not statistically significant. To eliminate any possible bias, additional regression analyses with adjustment for age were done. Since most of the data on frequencies of B-cell subsets were skewed, quantile regression models were used.

The first set of analyses was done to evaluate the effect of HIV infection (HIV status) on the frequencies of various B-cell subsets. This was done on all children. Subsequent analyses were restricted to the HIV-infected children to evaluate the predictive ability of viral loads, $CD4^+$ T-cell percentages and HAART treatment on the frequencies of various B-cell subsets. Since the three variables (i.e. viral loads, $CD4^+$ T-cell percentages and HAART treatment) showed multicollinearity, they were run on separate models. A generalized quantile regression model was also used to evaluate the effect of each variable after adjusting for all other variables.

B-cell subset	Age		HIV status	
	β Coefficient	P value	β Coefficient	P value
Naïve	0.10(0.06)	0.1	7.94(4.11)	0.056
Resting memory	0.04(0.02)	0.064	4.69(1.23)	<0.0005
Activated mature	-0.01(0.02)	0.557	-2.12(1.40)	0.127
Atypical memory	-0.08(0.03)	0.018	-5.69(2.20)	0.011
Plasmablasts	-0.02(0.01)	0.074	-0.54(0.78)	0.489
Immature/transitional	-0.03(0.03)	0.358	0.14(1.81)	0.939
IgD+ Resting memory	0.008(0.01)	0.438	1.90(0.63)	0.004
IgD- Resting memory	0.02(0.02)	0.313	3.48(1.08)	0.002

Table 3.18: Estimated change in frequencies of various B-cell subsets in relation to variations in age and HIV status (beta coefficients). Every B-cell subset was independently run in a quantile regression model containing HIV status and age. P values <0.05 were considered significant. Significant results are in red text.

B-cell subset	Age		Viral load	
	β Coefficient	P value	β Coefficient	P value
Naïve	-0.03(0.05)	0.500	-6.25(0.67)	<0.0005
Resting memory	-0.01(0.02)	0.683	-0.45(0.27)	0.105
Activated mature	0.00(0.02)	0.975	1.45(0.26)	<0.0005
Atypical memory	0.01(0.07)	0.886	3.53(0.88)	<0.0005
Plasmablasts	-0.01(0.01)	0.245	0.30(0.17)	0.079
Immature/transitional	-0.01(0.04)	0.889	0.48(0.56)	0.392
IgD+ Resting memory	0.00(0.00)	0.913	-0.31(0.14)	0.032
IgD- Resting memory	0.00(0.02)	0.982	-0.45(0.26)	0.086

Table 3.19: Estimated change in frequencies of various B-cell subsets in relation to variations in age and viral loads (beta coefficients) among the HIV-infected children only. Every B-cell subset was independently run in a quantile regression model containing age and viral load. P values <0.05 were considered significant. Significant results are in red text.

B-cell subset	Age		HAART	
	β Coefficient	P value	β Coefficient	P value
Naïve	0.02(0.14)	0.882	20.32(8.13)	0.015
Resting memory	0.01(0.02)	0.807	0.35(1.29)	0.789
Activated mature	-0.01(0.02)	0.739	-5.73(1.07)	<0.0005
Atypical memory	-0.025(0.08)	0.756	-12.65(4.40)	0.005
Plasmablasts	-0.01(0.01)	0.359	-2.63(0.80)	0.045
Immature/transitional	-0.00(0.04)	0.967	-2.31(2.14)	0.284
IgD+ Resting memory	0.00(0.00)	0.613	0.87(0.55)	0.117
IgD- Resting memory	0.00(0.01)	0.988	1.23(0.79)	0.127

Table 3.20: Estimated change in frequencies of various B-cell subsets in relation to variations in age and HAART treatment (beta coefficients) among the HIV-infected children. Every B-cell subset was independently run in a quantile regression model containing HAART treatment and age. P values <0.05 were considered significant. Significant results are in red text.

B-cell subset	Age		$CD4^+$ T-cell percentages	
	β Coefficient	P value	β Coefficient	P value
Naïve	0.09(0.12)	0.465	1.04(0.29)	0.001
Resting memory	-0.01(0.02)	0.611	0.10 (0.04)	0.015
Activated mature	-0.03(0.03)	0.331	-0.15(0.07)	0.049
Atypical memory	-0.08(0.07)	0.288	-0.56(0.17)	0.002
Plasmablasts	-0.03(0.01)	0.019	-0.04(0.03)	0.080
Immature/transitional	-0.02(0.03)	0.472	-0.10(0.08)	0.252
IgD+ Resting memory	0.01(0.01)	0.566	0.06(0.03)	0.039
IgD- Resting memory	-0.01(0.01)	0.487	0.05(0.03)	0.076

Table 3.21: Estimated change in frequencies of various B-cell subsets in relation to variations in age and $CD4^+$ T-cell percentage (beta coefficients) among the HIV-infected children. Every B-cell subset was independently run in a quantile regression model containing age and $CD4^+$ T-cell percentage. P values <0.05 were considered significant. Significant results are in red text.

B-cell subset	Age		HIV status		Viral load		$CD4^+$ T-cell percentages		HAART	
	β Coefficient	P value	β Coefficient	P value	β Coefficient	P value	β Coefficient	P value	β Coefficient	P value
Naïve	-0.06(0.05)	0.265	21.25(9.39)	0.026	-5.71(0.96)	<0.0005	0.17(0.14)	0.209	4.84(3.82)	0.208
Resting memory	0.02(0.02)	0.416	-3.43(3.97)	0.390	-0.02(0.41)	0.959	0.08(0.06)	0.185	0.15(1.6)	0.928
Activated mature	0.01(0.01)	0.690	-6.71(2.31)	0.005	1.20(0.23)	<0.0005	-0.00(0.04)	0.901	-2.32(0.96)	0.018
Atypical memory	0.01(0.05)	0.783	-16.33(8.51)	0.058	1.98(0.89)	0.027	-0.27(0.13)	0.040	-6.94(3.53)	0.052
Plasmablasts	0.00(0.01)	0.609	-3.04(1.61)	0.062	0.33(0.16)	0.045	0.02(0.03)	0.427	-1.08(0.66)	0.109
Immature/transitional	-0.01(0.03)	0.760	-6.87(5.27)	0.196	0.22(0.56)	0.691	-0.09(0.08)	0.256	-2.13(2.19)	0.333
IgD+ Resting memory	0.00(0.01)	0.750	-0.76(1.71)	0.660	-0.29(0.19)	0.133	0.01(0.02)	0.696	0.14(0.72)	0.841
IgD- Resting memory	0.02(0.02)	0.246	-1.35(3.59)	0.709	-0.11(0.41)	0.788	0.03(0.05)	0.507	0.43(1.51)	0.779

Table 3.22: Estimated change in frequencies of various B-cell subsets in relation to variations in age, HIV status, viral load, CD4+ T-cell percentage and HAART treatment (beta coefficients). Every B-cell subset was independently run in a generalized quantile regression model containing age, HIV status, viral load, $CD4^+$ T-cell percentage and HAART treatment. P values <0.05 were considered significant. Significant results are in red text.

When the predictive significance of HIV status on the frequencies of various B-cell subsets was assessed among all children after adjusting for age, HIV infection was a significant predictor of low frequencies of resting memory B cells, IgD^- resting memory B cells and IgD^+ resting memory B cells. Being HIV infected was also a significant predictor of high frequencies of atypical memory B cells. None of the other B-cell subsets were predictable by HIV status, probably due to heterogeneity in the frequencies of those subsets between different sub-groups (based on viraemia, $CD4^+$ T-cell percentages and HAART treatment) within the HIV-infected cohort.

When the predictive significance of viral load on the frequencies of various B-cell subsets was assessed among the HIV-infected children after adjusting for age, viral load was a significant predictor of low frequencies of naïve B cells and IgD^+ resting memory B cells. It also turned out to be a significant predictor of high frequencies of activated mature B cells and atypical memory B cells. Notably, there was a non-significant trend towards some predictive power of viral load on high frequencies of plasmablasts (P=0.079) and low frequencies of IgD^- resting memory B cells (P=0.086).

High $CD4^+$ T-cell percentages significantly predicted high frequencies of naïve B cells, resting memory B cells and IgD^+ resting memory B cells after adjusting for age in HIV-infected children. On the other hand, it predicted low frequencies of activated mature B cells and atypical memory B cells, suggesting that $CD4^+$ T-cell depletion in HIV could be associated with the expansion of some of these subsets.

Treatment with HAART, after adjusting for age, was a predictor of high frequencies of naïve B cells in HIV-infected children. It also predicted low frequencies of activated mature B cells, atypical memory B cells and plasmablasts.

In a generalized model that included all the predictor variables (i.e. age, HIV status, viral load, CD4+ T-cell percentage and HAART treatment), the effect of each predictor

variable after adjustment for all other variables was assessed. HIV infection was a significant predictor of low frequencies of naïve B cells and high frequencies of activated mature B cells. Viral load appeared to be the most important predictor of B-cell defects in most B-cell subsets. It significantly predicted low frequencies of naïve B cells and high frequencies of activated mature B cells, atypical memory B cells and plasmablasts. Interestingly, $CD4^+$ T-cell percentage was a significant predictor of only atypical memory B cells, suggesting that lymphopenia could directly affect atypical memory B cells compartment but has minimal direct effects on other subsets. Treatment with HAART was a significant predictor of low frequencies of activated mature B cells.

3.5 Discussion

In this study, the B-cell subset distribution in HIV-infected children was similar to that observed in HIV-infected adults but with minor differences [215, 236]. There was general expansion of B-cell subsets that are associated with generalized immune activation, namely activated mature B cells, atypical memory B cells and plasmablasts. This expansion appeared to be dependent mainly on viral loads, with low $CD4^+$ T-cell percentages and absence of HAART treatment also playing some role, suggesting that some viral factors could be directly activating B cells. Interestingly, treatment with HAART was associated with reduced frequencies of activated mature B cells among highly viraemic children.

HIV gp120 is one HIV factor that has been shown to polyclonally activate B cells via DC-SIGN and the B-cell receptor (BCR) [132, 225]. Moreover, BAFF, a cytokine that promotes B-cell survival, has been shown to be elevated in HIV viraemic patients and could be playing a role in maintaining some of these activated subsets [237]. In addition, ferritin released by macrophages in response to HIV Nef protein has also been

suggested to contribute to the pathogenic activation of B cells [133]. Therefore, the cause of expansion of these activated subsets is probably multifactorial.

Of note, unlike in HIV-infected adults and similar to one previous study in children, we did not detect differences in frequencies of immature/transitional B cells between the different groups of children [226, 234]. Any HIV-associated effects in this compartment could have been blunted by the fact that children usually have high frequencies of immature/transitional B cells that decrease with age [233], a factor that could lead to high variability and probably a large noise:signal ratio.

The low frequency of resting memory B cells, an observation that has been reported by previous studies in adults and children with HIV, was also seen here [84, 230-232, 234, 236]. It would be difficult to disentangle depletion of existing resting memory B cells from failure to induce new B-cell memory in children infected with HIV. This is because most HIV-infected children are infected early in life and therefore build their immune memory in the presence of the infection. In viraemic HIV-infected adults, depletion of existing B-cell memory has been shown to occur [228]. In addition, poor humeral response to vaccines has been reported in untreated HIV patients, suggesting that HIV viraemia could also impair induction of new B-cell memory [238]. Therefore, HIV-viraemic children could be experiencing both failure to induce adequate B-cell memory and depletion of the limited memory that they acquire. Notably, the low frequency of resting memory B cells was in both low viraemia and high viraemia children, suggesting some independence from viral loads as at that time.

Interestingly, we found significant interaction between age and level of viraemia with/without HAART with regard to accumulation of resting memory B cells. The same interaction was also observed between age and $CD4^+$ T-cell percentages with/without HAART. Normally, children are expected to acquire memory B cells with age due to the continued exposure to environmental and vaccine antigens as they advance in age.

Indeed, healthy children in this study showed an increase in frequencies of resting memory B cells with age. HIV-infected children who had low viraemia or high $CD4^+$ T-cell percentages too showed a similar expansion of the resting memory B-cell compartment with age, though the predicted frequencies remained consistently lower than those of healthy controls. However, HIV-infected children who had high viraemia or low $CD4^+$ T-cell percentages showed an age dependent (hence exposure dependent) decrease in frequencies of resting memory B cells. This finding suggests that, in the long run, having low viraemia could allow the HIV-infected children to slowly build up their B-cell memory. This emphasizes the possible advantages of putting children and even adults on HAART as soon as they are diagnosed with HIV. Indeed, children and adults on HAART with controlled viraemia or improved $CD4^+$ T-cell counts make better antibody responses to vaccines when compared with untreated patients [239]. Such observation can be extrapolated to the many other antigens that these people encounter in the environment over time, explaining the observed association of higher frequencies of resting memory B cells with older age among some of the groups of children.

The viral loads correlated with the frequencies of all B-cell subsets with the exception of resting memory B cells and immature/transitional B cells, suggesting that viral factors could be playing a major direct role in the deregulation of B-cell subset distribution. On the other hand, frequencies of resting memory B cells correlated positively with $CD4^+$ T-cell percentages, implying a strong connection between availability of T-cell help and the generation of memory B cells. However, a role for viral factors in depletion or prevention of generation of memory B cells cannot be ruled out since a good number of these children had a history of high viral loads that were controlled with HAART, hence abolishing any correlation that could have previously existed. Moreover, direct effect of viral factors could significantly affect frequencies of

resting memory B cells only at early days of infection when viral load is very high and germinal centers get extensively damaged. All the same, the significant inverse correlation between viral loads and $CD4^+$ T-cell percentages makes it difficult to disentangle the effect of each.

The significant inverse correlation between naïve B cells and the activated phenotypes suggests that the reduction in frequencies of naïve B cells could be as a result of the polyclonal activation. Indeed, some of the activated phenotypes could have been derived from the naïve B-cell compartment, leading to reduced frequencies of naïve B cells. For instance, atypical memory B cells have been shown to lie between classical memory B cells and naïve B cells with regard to replication histories and somatic hypermutations, suggesting that they cannot originate from the classical memory compartments hence leaving the naïve compartment as their likely source. However, this does not negate the possibility of them originating from a totally different compartment that is probably underrepresented in healthy individuals and only expanded in disease. The activating effect of gp120 on naïve B cells also supports the possibility that some of the activated phenotypes could be originating from the naïve compartment [132].

The significant correlations amongst the activated compartments suggest possible shared etiology or shared driving factors, though the generalized regression model suggests that atypical memory B cells are sensitive to $CD4^+$ T-cell percentages while activated mature B cells are not. Notably, the three activated phenotypes (activated mature B cells, atypical memory B cells and plasmablasts) and naïve B cells were significantly dependent on viral loads in the generalized regression model, suggesting a shared direct or indirect role of viral load in causing deregulation of all of them. The possibility of plasmablasts being derived from the activated mature B cells could explain the correlation between plasmablasts and activated mature B cells.

In summary, this study evaluated the subset distribution of B cells in HIV-infected children and how various factors relate with that subset distribution.

Chapter 4 Function of B cells in children infected with HIV

4.1 Literature review

Functional defects of B cells in HIV-infected adults have been described since the early days of the HIV epidemic. Most notable is hypergammaglobulinaemia, the polyclonal production of large amounts of immunoglobulins that are not targeted to any particular antigen and that are assumed to be of little use to the patient [122]. This is considered the classical indication of the aberrant activation of the B-cell compartment in untreated HIV. Other indicators of aberrant activation of B cells in untreated HIV adults are:

- The increased susceptibility to B-cell lymphomas [240].
- The production of auto reactive antibodies [241].
- Production of antibodies spontaneously when lymphocytes are cultured in the absence of stimulants [242].
- Terminal differentiation of B cells [123].

Paradoxically, despite the aberrant activation, B cells from untreated adults are relatively unresponsive to B-cell mitogens in vitro [122]. More importantly, untreated adults make poor B-cell responses to vaccines and pathogens. For instance, several studies have shown that they have low antibody levels against vaccines and pathogens [229, 243, 244]. Several other studies have shown depletion of antigen-specific memory B cells in such patients [84, 229]. However, little attention has been directed towards determination of antibodies' avidity maturation, with one study suggesting that the avidity of pre-existing anti-measles antibodies remains unaffected in HIV-infected adults [245].

The low levels of anti-vaccine antibodies and memory B cells in adults who were vaccinated before acquiring HIV suggests that HIV can deplete pre-existing B-cell responses. On the other hand, the poor response to vaccines administered to already

HIV-infected adults indicates that HIV-infection can also impair acquisition of new B-cell responses [238, 246].

Several factors could contribute to the aberrant activation of B cells in HIV. For instance, HIV infection is characterized by high concentrations of BAFF, a B-cell cytokine that could contribute to maintenance of activated B cells [237]. Gp120 has also been shown to cause polyclonal activation of B cells [132]. On the other hand, the poor B-cell responses to newly encountered antigens could be explained by the depletion of $CD4^+$ T cells that could be crucial for providing help to the responding B cells in an immune reaction. The ability of HIV patients' B cells to harness the available T-cell help has also been put to question [247]. In addition, some HIV factors, like HIV Nef protein, have been implicated in inhibiting isotype switching and therefore limiting the production of switched isotypes like IgG and IgA [25]. Induction of B-cell apoptosis in HIV infection has been shown to contribute to the loss of B-cell responses that were present before the individual was infected with HIV [248-251].

Controlling viraemia following initiation of HAART appears to reverse most functional B-cell defects that are attributable to polyclonal activation. As a result, most HAART-treated adults experience a reduction in levels of total immunoglobulins upon control of viraemia [252]. However, controlling viraemia does not restore antibody and memory B-cell responses to previously encountered vaccine antigens and pathogens, suggesting that such patients may remain susceptible to infections [215, 229]. Revaccination after successfully initiating HAART leads to attainment of protective antibody responses to most vaccines, though the magnitude of the responses is not as high as that in revaccinated healthy controls [253-256]. Partial restoration of the $CD4^+$ T-cell compartment and the elimination of detrimental HIV factors (e.g gp120 and Nef) could

explain the partial recovery in ability to make B-cell responses when viraemia is controlled with HAART.

Vertically infected children differ from adults in that their immune system develops in the presence of HIV. Since they mostly become infected in utero, at birth or during breast-feeding, they receive most childhood immunizations and infections when they are already HIV-infected, raising the question of whether HIV may impair induction of B-cell responses as well as deplete any induced responses. In addition, the interaction of the virus with an immature immune system could result in different outcomes, especially with regard to polyclonal activation. As a result, many studies have been conducted to investigate the effect of HIV-infection on the quantitative antibody response to vaccine antigens in HIV-infected children [245, 257-268]. However, few studies have been done to characterize avidity maturation and the acquisition/maintenance of memory B cells in these children [232, 245, 261].

Similar to adults, untreated HIV-infected children have shown elevated levels of total immunoglobulins [269]. In addition, they have low levels of specific antibodies to vaccine antigens; many of them fail to maintain protective titres despite going through the usual childhood immunization programs [245, 257-263]. They also have lower frequencies of antigen-specific memory B cells when compared with healthy children [232]. Unlike in adults, antibodies' avidity maturation to measles antigen is impaired in HIV-infected children [245, 261]. Controlling viraemia using HAART normalizes total immunoglobulin levels but does not restore prior anti-vaccine antibody responses [269, 270]. Subsequent revaccination induces protective titres that are long lasting, but its effect on antibody avidity maturation and generation of memory B cells has not been evaluated [257, 264-268]. Notably, initiation of HAART treatment in the first year of life has been shown to preserve the integrity of the memory B-cell compartment [232].

Untreated HIV patients have been shown to have disruptions of the structures of their germinal centres in various lymphoid organs [271]. Consequently, and in consensus with the literature reviewed above, it is possible that HIV affects most, if not all, of the processes that are attributable to the germinal centre reaction. In this chapter, I further investigate the effect of paediatric HIV infection on the outcomes of germinal centre reaction namely antibody avidity maturation, generation and maintenance of long-lived plasma cells (as measured by maintenance of plasma antibodies) and generation and maintenance of memory B cells. I evaluated B-cell responses to some components of routine childhood vaccines namely tetanus toxoid, diphtheria toxoid and measles virus lysate. I also evaluated responses to pneumococcal capsular polysaccharides to determine responses upon natural exposure to pneumococcal pathogens in the community. Of note, a pneumococcal conjugate vaccine was introduced in the community during this study. Therefore, the analysis of pneumococcal responses in this chapter was limited to the children that had not received the pneumococcal vaccine.

Vaccine	Full name	Components	Age of administration
BCG	Bacillus Calmette–Guérin	Live attenuated *Mycobacterium bovis*	At birth
OPV	Oral polio vaccine	Live attenuated polio virus	At birth, 6 weeks, 10 weeks and 14 weeks
DPT-HepB-Hib	Diphtheria, Pertussis, Tetanus, Hepatitis B and Hemophilus influenza type B	Diphtheria toxoid, Tetanus toxoid, *Bordetella pertussis* toxins and adhesins, Hepatitis B surface antigen, Hemophilus influenza polysaccharides conjugated to carrier proteins	6 weeks, 10 weeks and 14 weeks
PCV 10	Decavalent pneumococcal conjugate vaccine	Pneumococcal capsular polysaccharides conjugated to carrier proteins	6 weeks, 10 weeks and 14 weeks
Measles vaccine		Live attenuated measles virus	9 months

Table 4.1: Summary of the routine childhood vaccines that are part of the Kenya Expanded Programme on Immunisation (KEPI). The vaccines components that were evaluated are in red text.

4.2 Objectives

I determined the function of B cells in children infected with HIV with regard to various antigens. The specific objectives were:

1. To determine the levels of antibodies to common childhood vaccines and infections in HIV-infected children.
2. To determine the avidity of antibodies to common childhood vaccines and infections in HIV-infected children.
3. To determine the frequencies of memory B cells to common childhood vaccines and infections in HIV-infected children.

4.3 Results

The population characteristics of the HIV-infected children and community controls that were studied in this chapter are shown in table 4.1.

	Value	P value (versus community controls)
Number (N)	174	
Community controls	58	
HIV-infected	116	
Low viraemia	64	
High viraemia	52	
High $CD4^+$ T-cell percentage	49	
Low $CD4^+$ T-cell percentage	67	
Median age in months	55 (35-80)	
Community controls	55 (43-81)	
HIV-infected	53 (31-79)	0.2617 [a]
Low viraemia	58 (29-86)	0.5623 [a]
High viraemia	49 (33-72)	0.1580 [a]
High $CD4^+$ T-cell percentage	59 (35-82)	0.7386 [a]
Low $CD4^+$ T-cell percentage	47 (31-71)	0.0758 [a]
% Female (n)	50 (87)	
Community controls	48 (28)	
HIV-infected	51 (59)	0.707 [b]
Low viraemia	56 (35)	0.423 [b]
High viraemia	46 (24)	0.824 [b]
High $CD4^+$ T-cell percentage	58 (38)	0.300 [b]
Low $CD4^+$ T-cell percentage	43 (21)	0.575 [b]
% on HAART(n)	N/A	
Community controls	N/A	
HIV-infected	61 (71)	
Low viraemia	78 (50)	
High viraemia	40 (21)	
High $CD4^+$ T-cell percentage	73 (49)	
Low $CD4^+$ T-cell percentage	45 (22)	
Viral load, log10 RNA copies/mL	N/A	
Community controls	N/A	
HIV-infected	3.4 (0-4.6)	
Low viraemia	1.4 (0-3.2)	

High viraemia	4.7 (4.4-5.2)	
High $CD4^+$ T-cell percentage	2.7 (0-4.1)	
Low $CD4^+$ T-cell percentage	4.4 (3.2-4.9)	
$CD4^+$ T cells (Percentage)	27.7 (19.6-34.5)	
Community controls	33.8 (28.1-39.3)	
HIV-infected	26.2 (18.1-32.9)	0.0001 [a]
Low viraemia	29.4 (22.5-34.5)	0.0069 [a]
High viraemia	21.3 (9.4-27.4)	<0.0005 [a]
High $CD4^+$ T-cell percentage	32.0 (28.1-37.8)	0.3757 [a]
Low $CD4^+$ T-cell percentage	16.5 (8.7-22.0)	<0.0005 [a]
% PHiD-CV vaccinated (n)	28 (49)	
Community controls	15 (9)	
HIV-infected	35 (40)	0.007 [b]
Low viraemia	38 (24)	0.005 [b]
High viraemia	31 (16)	0.049 [b]
High $CD4^+$ T-cell percentage	42 (28)	0.001 [b]
Low $CD4^+$ T-cell percentage	26 (12)	0.202 [b]

Table 4.1: Baseline characteristics of the children who participated in the evaluation of B-cell function in HIV-infected children. Values shown are medians (inter-quartile range) unless otherwise stated. Statistical tests used|: [a]Wilcoxon rank-sum test (Mann Whitney test), [b]Chi-squared test.

However, some of the children were not considered in the analysis in this chapter. Fourty nine children were omitted from the evaluation of anti-pneumococcal responses because they had participated in the community catch-up campaign for introduction of a pneumococcal conjugate vaccine, Synflorix®, which was introduced into the childhood immunization schedule when this study was ongoing. As a result, some older children in the community received the vaccine in the community. Since the evaluation of pneumococcal responses in this study was aimed at assessing the responses from natural exposure to pneumococcal antigens, children who had received the vaccine were left out in the analysis of responses to pneumococcal capsular polysaccharide in this chapter. Furthermore, two of the polysaccharides in the vaccine were conjugated to tetanus toxoid and diphtheria toxoid. The vaccine therefore acted as a booster to pre-existing anti-tetanus and anti-diphtheria responses. Since the analyses of the anti-tetanus and anti-diphtheria responses in this chapter were aimed at describing the responses that

were attributable to the DPT vaccination in the first months of life, such children who received the pneumococcal conjugate vaccine in the community were also excluded from the analyses. Eleven children whose DPT vaccination information was not available were also excluded from the analyses on tetanus toxoid and diphtheria toxoid responses. Other children were omitted from the various analyses because their samples had not been collected at the time when the assays were carried out.

After stratification of the 174 total children on the basis of viral loads and $CD4^+$ T-cell percentages, differences in age were revealed amongst the various HIV-infected subgroups and community controls. Initial statistical analyses were done using univariate nonparametric tests, mainly Wilcoxon rank-sum test (Mann-Whitney test). Subsequent analyses were performed using multivariate regression models whereby adjustments for age and other covariates were done.

4.3.1 Quantities of antibodies to vaccines and natural infections

Similar to previous reports, HIV-infected children had lower levels of IgG to vaccine antigens when compared with community control children. The whole HIV-infected cohort had lower median levels of IgG against tetanus toxoid (47.5 [IQR, 29.6 -112.4] AU versus 174.3 [IQR, 52.5 -653.7] AU, P=0.0122), diphtheria toxoid (230.9 [IQR, 144.2-349.8] AU versus 890.5 [IQR, 531.4-1537.9] AU, P<0.00005) and measles antigen (19.3 [IQR, 10.4-44.6] AU versus 51.7 [IQR, 25.5-103.5] AU, P<0.00005) when compared with community controls. Following further classification of the HIV-infected children into high viraemia and low viraemia groups, the two groups still had comparably low median levels of anti-tetanus toxoid (high viraemia, 46.5 [IQR, 28.5-87.2] AU P=0.0236; low viremia, 54.0 [IQR, 26.3-117.8] AU, P=0.0281), anti-diphtheria toxoid (high viraemia, 244.0 [IQR, 175.5-422.4] AU P<0.00005; low viraemia, 205.0 [IQR, 133.9-327.0] AU P<0.00005) and anti-measles (high viraemia, 18.5 [IQR, 11.2-35.1] AU P=0.0001; low viraemia, 21.6 [IQR, 9.7-48.0] AU P=0.0005) IgG when compared with community controls, suggesting that the level of viraemia at that time did not influence the acquisition/maintenance of plasma anti-vaccine IgG concentrations. Stratification of the HIV-infected children on the basis of $CD4^+$ T-cell percentages yielded similar results with both low and high $CD4^+$ T-cell percentage groups having low anti-tetanus toxoid (low $CD4^+$ T-cell percentage group, 46.8 [IQR, 30.1-113.0] AU, P=0.0222; High $CD4^+$ T-cell percentage group, 51.3 [IQR, 52.5-653.7] AU, P=0.0296), anti-diphtheria toxoid (low $CD4^+$ T-cell percentage group, 219.5 [IQR, 175.1-318.4] AU, P<0.00005; High $CD4^+$ T-cell percentage group, 244.7 [IQR, 141.3-427.7] AU, P<0.00005) and anti-measles (low $CD4^+$ T-cell percentage group, 18.6 [IQR, 11.6-45.0] AU, P=0.0005; High $CD4^+$ T-cell percentage group, 19.9 [IQR, 9.8-43.7] AU, P=0.0002) IgG when compared with community controls. Interestingly, there

were no differences between the community controls and the HIV-infected children with regard to IgG against pneumococcal capsular polysaccharides.

Further stratification of the high and low viraemia children on the basis of their HAART-treatment status did not reveal any differences between the HAART-naïve and the HAART-treated children with regard to levels of IgG against any of the antigens. Notably, only the HAART-treated subgroups had lower quantities of anti-TT IgG while all subgroups had lower anti-DT IgG when compared with community controls. In addition, all subgroups, except the low viraemia sub-group, had lower anti-measles IgG when compared with community controls. None of the sub-groups differed from community controls with regard to IgG levels against pneumococcal capsular polysaccharides.

Further stratification of the low and high $CD4^+$ T-cell percentage groups on the basis of treatment also revealed no differences between HAART-naïve and HAART-treated subgroups within both low and high $CD4^+$ T-cell percentage groups with regard to all antigens. However, the HAART-treated subgroups showed lower ant-TT IgG levels when compared with community controls. All subgroups had lower levels of IgG against DT and measles when compared with community controls. None differed from community controls with regard to IgG against pneumococcal capsular polysaccharides.

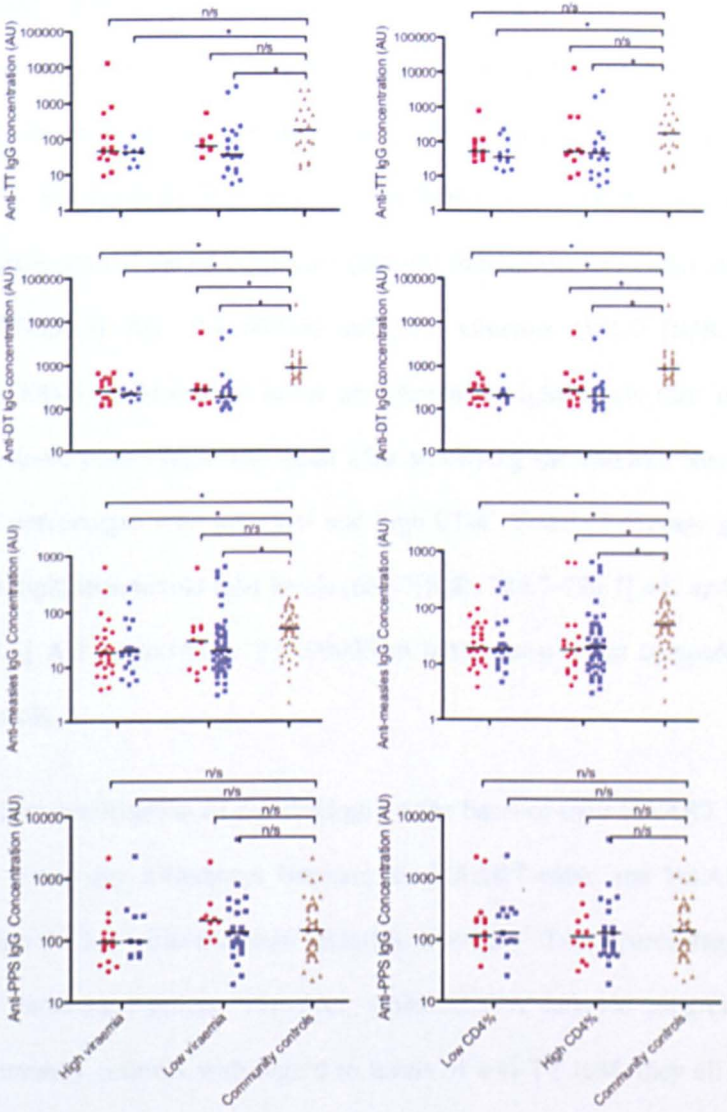

Figure 4.1: Plasma concentrations in arbitrary units (AU) of IgG against tetanus toxoid (TT), diphtheria toxoid (DT), measles and pneumococcal capsular polysaccharides (PPS). Blue symbols represent children who were treated with HAART, red symbols represent children who were HAART-naïve and brown symbols represent the community controls. Horizontal line shows the median concentration for each sub-group. High viraemia group: >/=5000 RNA copies/ml, low viraemia group: < 5000 RNA copies/ml, low CD4 percentage group: <25% and high CD4 percentage group: >/=25%. Statistical test used: Wilcoxon rank-sum test. * represents $p<0.05$.

Notably, plasma IgM levels against tetanus toxoid were similar in HIV-infected and community control children. However, plasma IgM levels against diphtheria toxoid were lower in the HIV-infected cohort when compared with community controls (594.5 [IQR, 393.5-828.8] AU versus 2276.6 [IQR, 1135.5-3409.1] AU P<0.00005). Further classification of the HIV-infected children showed that both high viraemia (649.3 [IQR, 375.7-830.3] AU, P<0.00005) and low viraemia (581.0 [IQR, 412.6-844.5] AU, P<0.00005) children had lower anti-diphtheria IgM levels than community controls. The same observation was made after stratifying the children based on their $CD4^+$ T-cell percentages with both low and high $CD4^+$ T-cell percentage groups having lower anti-diphtheria toxoid IgM levels (651.7[IQR, 384.7-776.7] AU and 554.7 [IQR, 412.6-844.5] AU respectively, P<0.00005 in both cases) when compared with community controls.

Further stratification of the children on the basis of their HAART treatment status did not reveal any differences between the HAART-naïve and HAART treated children within the low viraemia, high viraemia, low $CD4^+$ T-cell percentage and high $CD4^+$ T-cell percentage groups. However, while all HIV-infected subgroups were similar to community controls with regard to levels of anti-TT IgM, they all had lower levels of anti-DT IgM than community controls.

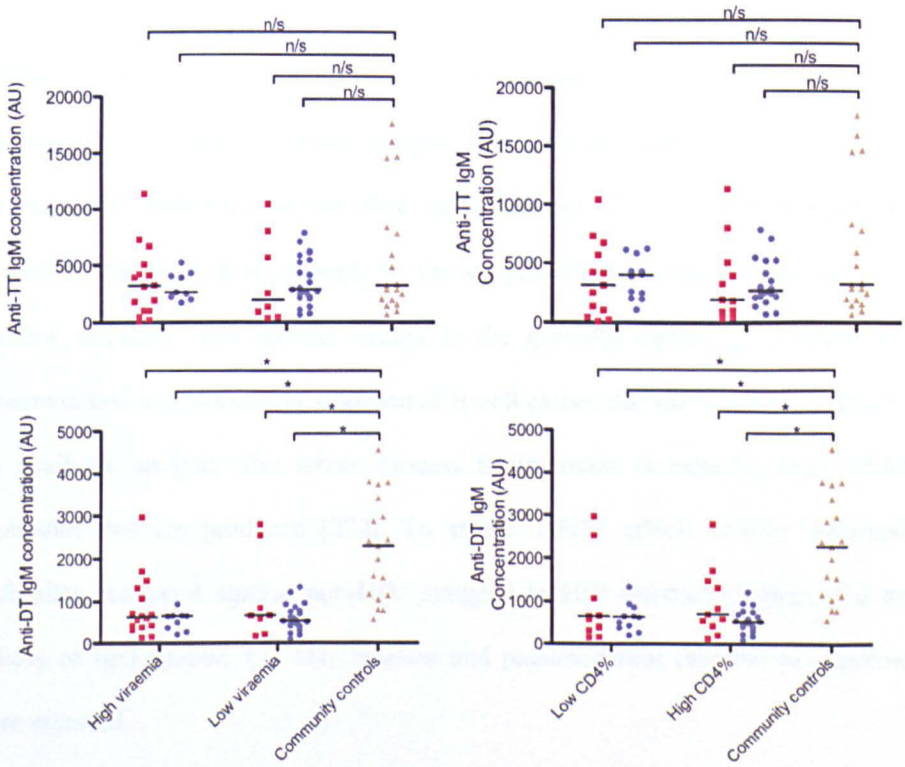

Figure 4.2: Plasma concentrations in arbitrary units (AU) of IgM against tetanus toxoid (TT) and diphtheria toxoid (DT). Blue symbols represent children who were treated with HAART, red symbols represent children who were HAART naïve and brown symbols represent the community controls. Horizontal line shows the median concentration for each sub-group. High viraemia group: >/=5000 RNA copies/ml, low viraemia group: < 5000 RNA copies/ml, low CD4 percentage group: <25% and high CD4 percentage group : >/=25%. Statistical test used: Wilcoxon rank-sum test. * represents $p<0.05$.

In summary, HIV-infected children had lower vaccine-derived antibody levels when compared with community controls. However, they were not any different with regard to naturally acquired antibodies against pneumococcal antigens.

4.3.2 Avidities of IgG to vaccine antigens and natural infections

Antibody avidity index is a measure of the strength of the interaction between an antibody molecule and its cognate antigen. Avidity maturation is the process by which the produced antibodies are modified in the course of an immune reaction so that antibodies that bind more strongly to the antigen are produced at later stages of the immune response. The process occurs in the germinal centre. It involves somatic hypermutation and Darwinian selection of B-cell clones that interact more strongly with the available antigen. The whole process is important in ensuring high quality of antibodies that are produced [272]. To assess if HIV affects avidity maturation of antibodies produced against non-HIV antigens in HIV-infected children, the avidity indices of IgG against TT, DT, measles and pneumococcal capsular polysaccharides were assessed.

The avidity indices of antibodies against tetanus toxoid were lower among the HIV infected children (0.22 [IQR, 0.14-0.34]) when compared with community control children (0.38 [IQR, 0.27-0.51], P=0.0143). Further stratification of the HIV-infected children revealed that the impairment was in both high viraemia group (0.21 [IQR, 0.15-0.32], P=0.0152) and low $CD4^+$ T-cell percentage group (0.19 [IQR, 0.13-0.32], P=0.0110). The same effect was also observed for avidity indices of antibodies against diphtheria toxoid when the whole HIV-infected cohort (0.34 [IQR, 0.28-0.43], P=0.0006), high viraemia group (0.37 [IQR, 0.29-0.43], P=0.0010), low viraemia group (0.34 [IQR, 0.26-0.44], P=0.0042), low $CD4^+$ T-cell percentage group (0.37 [IQR, 0.30-0.44], P=0.0016) and high $CD4^+$ T-cell percentage group (0.32 [IQR, 0.26-0.44], P=0.0028) were compared with community controls (0.51 [IQR, 0.41-0.57]). The avidity indices against measles antigen were also lower in the whole HIV-infected cohort (0.22 [IQR, 0.16-0.27], P<0.00005), low viraemia (0.22 [IQR, 0.16-0.30],

P=0.0002), high viraemia (0.21 [IQR, 0.16-0.24], P<0.00005), low $CD4^+$ T-cell percentage (0.21 [IQR, 0.16-0.28], P=0.0002) and high $CD4^+$ T-cell percentage (0.22 [IQR, 0.16-0.26], P<0.00005) groups when compared with community controls (0.30 [IQR, 0.23-0.38]), suggesting that HIV infection generally impairs the process of avidity maturation to routine vaccines in children. Notably, the HIV-infected children did not differ from community controls with regard to anti-pneumococcal capsular polysaccharides IgG avidity indices.

Further stratification of the children on the basis of their HAART treatment status did not reveal any differences between the HAART-naïve and HAART-treated children within the low viraemia, high viraemia, low $CD4^+$ T-cell percentage and high $CD4^+$ T-cell percentage groups with regard to avidity indices against any of the antigens. For TT, the HAART-treated subgroups had lower avidity indices when compared with community controls. For DT, all subgroups, except the low viraemia HAART-naïve and low $CD4^+$ T-cell percentage HAART-naïve subgroups, had lower avidity indices when they were compared with community controls. All subgroups, except the low viraemia HAART-naïve subgroup, had lower avidity indices of anti-measles IgG. However, none of the sub-groups differed from community controls with regard to avidity indices of anti-pneumococcal IgG.

In summary, HIV-infected children had lower avidity indices for IgG against vaccine antigens when compared with community controls. However, the same was not observed with regard to avidity indices for IgG against pneumococcal antigens.

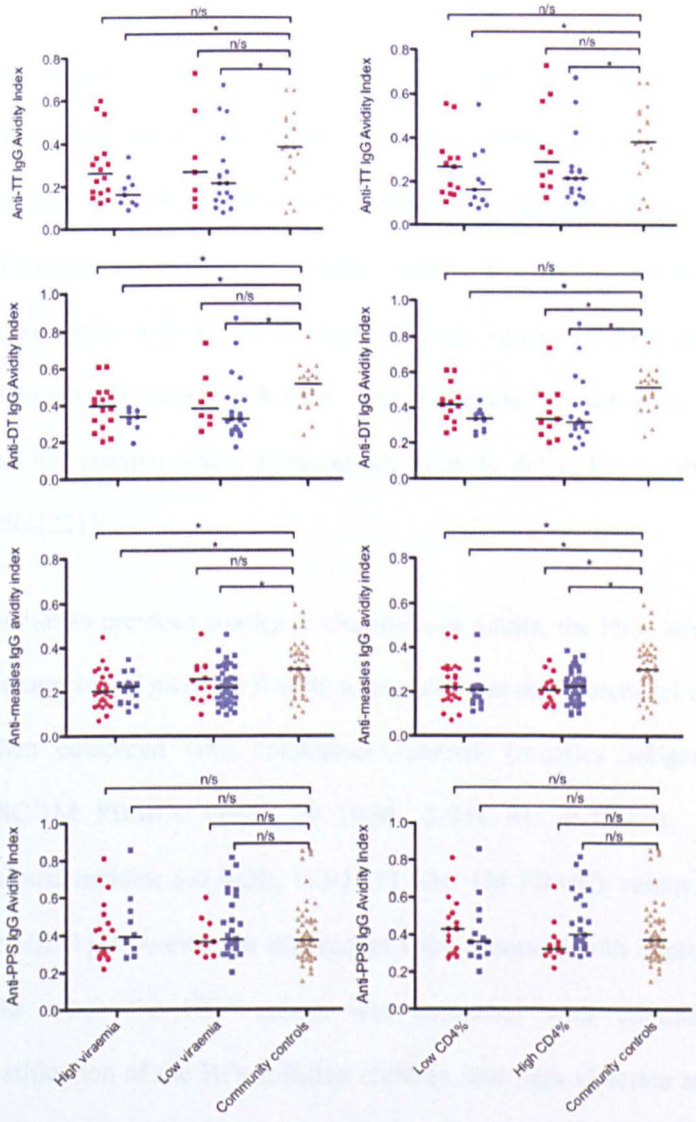

Figure 4.3: Avidity indices of plasma IgG against tetanus toxoid (TT), diphtheria toxoid (DT), measles and pneumococcal capsular polysaccharides (PPS). Blue symbols represent children who were treated with HAART, red symbols represent children who were HAART naïve and brown symbols represent the community controls. Horizontal line shows the median concentration for each sub-group. High viraemia group: >/=5000 RNA copies/ml, low viraemia group: < 5000 RNA copies/ml, low CD4 percentage group: <25% and high CD4 percentage group: >/=25%. Statistical test used: Wilcoxon rank-sum test. * represents $p<0.05$.

4.3.3 Frequencies of memory B cells to vaccine antigens

One of the products of a germinal centre reaction is long-lived memory B cells against the cognate antigen. Such memory B cells enable the individual to mount a quicker and stronger antibody response in the event of re-exposure to the same antigen. Assessment of frequencies of antigen specific memory B cells is done by first activating PBMCs polyclonally with B-cell mitogens that are known to drive terminal differentiation of preferentially memory B cells. The polyclonal activation is followed by an antigen specific enzyme linked immunospot assay to detect the number of antibody secreting cells [221].

Similar to previous studies in children and adults, the HIV-infected children had lower frequencies of memory B cells to measles and pneumococcal capsular polysaccharides when compared with community controls (measles antigen; 3.8 [IQR, 0.1-17.5] ASC/1M PBMCs versus 20 [IQR, 5-35] AU P=0.0051, pneumococcal capsular polysaccharides; 5.0 [IQR, 0.0-17.5] ASC/1M PBMCs versus 13.8 [IQR, 6.3-40] AU P=0.0281). However, no differences were observed with regard to anti-TT memory B cells when the HIV cohort was compared with community controls. Further stratification of the HIV-infected children into high viraemia and low viraemia groups revealed that the low viraemia group had low frequencies of memory B cells against measles antigen when compared with community controls. Stratification into high and low $CD4^+$ T-cell percentage groups revealed that both groups had lower frequencies of anti-measles memory B cells when compared with community controls. For pneumococcal antigens, both low viraemia and high $CD4^+$ T-cell percentage groups had low frequencies of memory B cells when compared with community controls. No differences were observed between the HIV-infected groups and community controls with regard to anti-TT memory B cells.

When further stratification of the children on the basis of their treatment with HAART was done, the HAART-treated children with low viraemia had higher frequencies of memory B cells against measles antigen and pneumococcal capsular polysaccharides when compared with HAART-naïve children who also had low viraemia. No other differences were observed between HAART-naïve and HAART-treated children within any other groups with regard to any of the antigens. Notably, for measles antigen, all HAART-naïve subgroups had lower frequencies of memory B cells when compared with community controls. For TT, only the HAART-treated children who had low $CD4^+$ T-cell percentages showed lower frequencies of anti-TT memory B cells when compared with community controls. For pneumococcal antigens, the high viraemia HAART-treated, low viraemia HAART-naïve and low $CD4^+$ T-cell percentage HAART-naïve groups had lower frequencies of anti-pneumococcal memory B cells when compared with community controls.

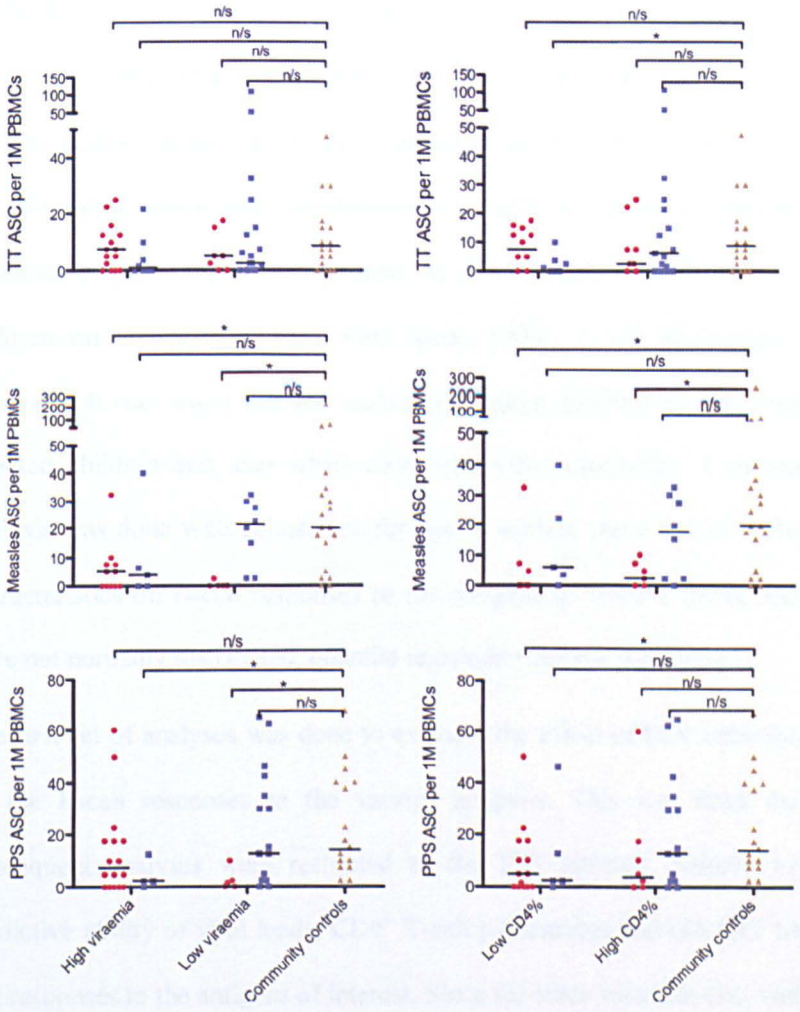

Figure 4.4: Frequencies of antigen-specific IgG memory B cells in peripheral blood against tetanus toxoid (TT), measles and pneumococcal capsular polysaccharides (PPS) expressed as IgG antibody-secreting cells(ASC) per 1 million PBMCs. Blue symbols represent children who were treated with HAART, red symbols represent children who were HAART naïve and brown symbols represent the community controls. Horizontal line shows the median concentration for each sub-group. High viraemia group: >/=5000 RNA copies/ml, low viraemia group: < 5000 RNA copies/ml, low CD4 percentage group: <25% and high CD4 percentage group : >/=25%. Statistical test used: Wilcoxon rank-sum test. * represents $p<0.05$.

Multivariate regression analysis with adjustment for age

B-cell responses to vaccine antigens and natural infections vary with age. Responses against vaccine antigens are expected to wane over time, as the children grow older. On the other hand, responses to environmental antigens and natural infections are expected to increase over time with exposure, as children grow older. After stratifying the children on the basis of their viral loads, $CD4^+$ T-cell percentages and HAART treatment, it was noted that the various subgroups differed in age amongst the HIV-infected children and also when compared with community. Consequently, further analysis was done with adjustment for age to analyse the effect of various population characteristics on B-cell responses to the antigens of interest. Since most of the data were not normally distributed, quantile regression models were used.

The first set of analyses was done to evaluate the effect of HIV infection (HIV status) on the B-cell responses to the various antigens. This was done on all children. Subsequent analyses were restricted to the HIV-infected children to evaluate the predictive ability of viral loads, $CD4^+$ T-cell percentages and HAART treatment on B-cell responses to the antigens of interest. Since the three variables (i.e. viral loads, $CD4^+$ T-cell percentages and HAART treatment) showed multicollinearity, they were first run on separate models then later run together on a generalized model.

When the predictive ability of HIV status was evaluated with adjustment for age alone, HIV infection was a significant predictor of low IgG responses against TT, DT and measles antigen. It also significantly predicted low levels of anti-DT IgM. In addition, HIV significantly predicted low avidity indices for IgG against TT, DT and measles antigen and low frequencies of memory B cells against measles antigen. It did not predict any B-cell responses against pneumococcal capsular polysaccharides,

suggesting that HIV infection does not affect the acquisition of B-cell responses against environmental pneumococcal antigens.

Interestingly, when the effect of viral load was assessed among the HIV-infected children after adjustment for age, viral load came out as a significant negative predictor of IgG against pneumococcal capsular polysaccharides, suggesting that even though HIV-infection does not affect acquisition of anti-pneumococcal responses, viral load does. The other B-cell responses were not affected by viral load at the time, suggesting that it had no effect for as long as a child was infected.

When the effect of $CD4^+$ T-cell percentage was assessed with adjustment for age among the HIV-infected children, it did not affect any of the B-cell responses, suggesting that $CD4^+$ T-cell percentages at the chronic phase of HIV-infection do not influence responses to historically encountered antigens.

Surprisingly, HAART treatment was a predictor of low anti-DT IgM and low avidity indices of IgG against TT after adjustment for age, probably because the children on HAART are the same ones whose disease had progressed more in the first months of life, hence them being put on HAART. HAART treatment did not predict any other B-cell response.

In the generalized quantile regression model where age, HIV status, viral loads, $CD4^+$ T-cell percentages and HAART treatment were included, the effect of each variable with adjustment for the other variables was assessed. HIV infection was still a significant predictor of low levels of IgG against TT, DT and measles as well as low levels of IgM against DT. It also significantly predicted for low avidity indices of IgG against DT and measles antigen, but not TT. Interestingly, it did not significantly predict frequencies of memory B cells against any of the antigens. Viral load and $CD4^+$

T-cell percentages did not significantly predict any of the B-cell responses. HAART treatment significantly predicted for higher frequencies of anti-measles memory B cells, suggesting that treatment with HAART in children can indeed influence memory B-cell responses against some vaccines. Notably, age was a significant predictor of anti-TT IgG levels and anti-pneumococcal IgG avidity indices, suggesting that age could only influence some of the B-cell responses.

B-cell response	HIV status		Age(Months)	
	β Coefficient	P value	β Coefficient	P value
Anti-TT IgG	-144.83(13.95)	0.000	-0.72(0.22)	0.002
Anti-DT IgG	-720.13(79.84)	0.000	-1.19(1.23)	0.338
Anti-Measles IgG	-36.22(4.40)	0.000	-0.18(0.07)	0.011
Anti-PPS IgG	-31.25(28.87)	0.282	0.87(0.52)	0.098
Anti-TT IgM	-272.82(1298.99)	0.834	8.93(20.50)	0.664
Anti-DT IgM	-1596.00(158.19)	0.000	2.69(2.50)	0.286
Anti-TT IgG AI	-0.17(0.06)	0.006	-0.00(0.00)	0.832
Anti-DT IgG AI	-0.17(0.05)	0.000	-0.00(0.00)	0.389
Anti-Measles IgG AI	-0.08(0.02)	0.000	0.00(0.00)	0.996
Anti-PPS IgG AI	-0.01(0.04)	0.890	0.00(0.00)	0.204
Anti-TT IgG ASC/1MPBMCs	-5.23(3.24)	0.112	-0.04(0.05)	0.473
Anti-Measles IgG ASC/1MPBMCs	-18.81(6.01)	0.003	-0.05(0.10)	0.589
Anti-PPS IgG ASC/1MPBMCs	-10.00 (6.73)	0.143	0.00(0.10)	1.000

Table 4.2: Estimated change in B-cell responses in relation to variations in age and HIV status (beta coefficients). Every B-cell response was independently run in a quantile regression model containing HIV status and age. P values <0.05 were considered significant. TT-tetanus toxoid; DT-diphtheria toxoid; PPS-pneumococcal capsular polysaccharides; AI-avidity index; ASC/1MPBMCs-antibody secreting cells per 1 million PBMCs. Significant results are in red text.

B-cell response	Viral load		Age(Months)	
	β Coefficient	P value	β Coefficient	P value
Anti-TT IgG	-1.22(3.61)	0.736	-0.79(0.30)	0.011
Anti-DT IgG	10.91(15.31)	0.479	-1.15(1.24)	0.358
Anti-Measles IgG	-0.42(0.95)	0.662	-0.21(0.06)	0.001
Anti-PPS IgG	-10.68(4.93)	0.034	-0.15(0.39)	0.700
Anti-TT IgM	311.98(225.19)	0.172	27.00(18.31)	0.146
Anti-DT IgM	1.75(29.56)	0.953	0.85(2.45)	0.731
Anti-TT IgG AI	0.00(0.01)	0.972	-0.00(0.00)	0.819
Anti-DT IgG AI	0.01(0.01)	0.314	-0.00(0.00)	0.704
Anti-Measles IgG AI	-0.00(0.00)	0.811	0.00(0.00)	0.934
Anti-PPS IgG AI	0.00(0.01)	0.794	-0.00(0.00)	0.105
Anti-TT IgG ASC/1MPBMCs	0.28(0.92)	0.761	-0.06(0.08)	0.469
Anti-Measles IgG ASC/1MPBMCs	-0.34(1.41)	0.809	0.20(0.11)	0.096
Anti-PPS IgG ASC/1MPBMCs	0.70(1.75)	0.691	-0.06(0.11)	0.596

Table 4.3: Estimated change in B-cell responses in relation to variations in age and viral load (beta coefficients) among the HIV-infected children only. Every B-cell response was independently run in a quantile regression model containing age and viral load. P values <0.05 were considered significant. TT-tetanus toxoid; DT-diphtheria toxoid; PPS-pneumococcal capsular polysaccharides; AI-avidity index; ASC/1MPBMCs-antibody secreting cells per 1 million PBMCs. Significant results are in red text.

B-cell response	$CD4^+$ T-cell percentage		Age(Months)	
	β Coefficient	P value	β Coefficient	P value
Anti-TT IgG	0.30(0.51)	0.551	-0.74(0.22)	0.001
Anti-DT IgG	-1.34(2.51)	0.597	-1.21(1.09)	0.272
Anti-Measles IgG	-0.08(0.21)	0.713	-0.19(0.08)	0.023
Anti-PPS IgG	-1.27(1.90)	0.507	0.70(0.77)	0.363
Anti-TT IgM	-30.14(27.36)	0.276	20.00(11.75)	0.095
Anti-DT IgM	3.22(5.76)	0.578	2.49(2.50)	0.324
Anti-TT IgG AI	0.00(0.00)	0.502	-0.00(0.00)	0.743
Anti-DT IgG AI	-0.00(0.00)	0.207	-0.00(0.00)	0.486
Anti-Measles IgG AI	0.00(0.00)	0.206	0.00(0.00)	0.635
Anti-PPS IgG AI	-0.00(0.00)	0.565	-0.00(0.00)	0.075
Anti-TT IgG ASC/1MPBMCs	0.07(0.10)	0.486	-0.06(0.04)	0.156
Anti-Measles IgG ASC/1MPBMCs	0.11(0.26)	0.677	0.16(0.12)	0.204
Anti-PPS IgG ASC/1MPBMCs	-0.10(0.31)	0.759	-0.06(0.11)	0.610

Table 4.4: Estimated change in B-cell responses in relation to variation in age and $CD4^+$ T-cell percentage (beta coefficients) among the HIV-infected children only. Every B-cell response was independently run in a quantile regression model containing age and $CD4^+$ T-cell percentage. P values <0.05 were considered significant. TT-tetanus toxoid; DT-diphtheria toxoid; PPS-pneumococcal capsular polysaccharides; AI-avidity index; ASC/1MPBMCs-antibody secreting cells per 1 million PBMCs. Significant results are in red text.

B-cell response	HAART		Age(Months)	
	β Coefficient	P value	β Coefficient	P value
Anti-TT IgG	2.62(11.05)	0.813	-0.71(0.22)	0.002
Anti-DT IgG	-37.44(83.82)	0.657	-0.85(1.66)	0.611
Anti-Measles IgG	2.50(4.24)	0.557	-0.20(0.07)	0.004
Anti-PPS IgG	1.12(31.7)	0.972	0.51(0.57)	0.371
Anti-TT IgM	-597.18(941.24)	0.529	13.89(18.22)	0.450
Anti-DT IgM	-246.35(121.72)	0.048	4.61(2.44)	0.064
Anti-TT IgG AI	-0.11(0.05)	0.050	0.00(0.00)	0.473
Anti-DT IgG AI	-0.07(0.05)	0.141	-0.00(0.00)	0.714
Anti-Measles IgG AI	0.00(0.02)	0.907	0.00(0.00)	0.899
Anti-PPS IgG AI	0.07(0.05)	0.186	0.00(0.00)	0.025
Anti-TT IgG ASC/1MPBMCs	-0.67(3.03)	0.826	-0.04(0.06)	0.522
Anti-Measles IgG ASC/1MPBMCs	8.43(6.09)	0.180	0.12(0.11)	0.324
Anti-PPS IgG ASC/1MPBMCs	7.5(5.99)	0.218	-0.00(0.10)	1.000

Table 4.5: Estimated change in various B-cell responses in relation to variations in age and HAART treatment (beta coefficients) among the HIV-infected children. Every B-cell response was independently run in a quantile regression model containing HAART treatment and age. P values <0.05 were considered significant. TT-tetanus toxoid; DT-diphtheria toxoid; PPS-pneumococcal capsular polysaccharides; AI-avidity index; ASC/1MPBMCs-antibody secreting cells per 1 million PBMCs. Significant results are in red text.

B-cell response	Age		HIV status		Viral load		$CD4^+$ T-cell %		HAART	
	β Coefficient	P value	β Coefficient	P value	β Coefficient	P value	β Coefficient	P value	β Coefficient	P value
Anti-TT IgG	-0.82(0.31)	0.010	-163.42(47.04)	0.001	-1.30(4.78)	0.786	0.34(0.73)	0.645	-8.25(19.98)	0.681
Anti-DT IgG	-1.45(1.33)	0.280	-855.6(205.8)	0.000	4.58(20.32)	0.822	-0.98(3.20)	0.760	12.05(87.53)	0.891
Anti-Measles IgG	-0.22(0.11)	0.058	-41.38(18.69)	0.029	0.11(1.89)	0.952	-0.08(0.31)	0.794	4.60(7.76)	0.554
Anti-PPS IgG	0.76(0.96)	0.433	-77.11(153.87)	0.618	-3.78(15.67)	0.810	-1.98(2.45)	0.423	0.31(61.76)	0.996
Anti-TT IgM	19.25(20.64)	0.354	665.71(3148.91)	0.833	72.56(320.34)	0.822	-32.91(49.32)	0.507	496.34(1354.80)	0.715
Anti-DT IgM	3.69(3.32)	0.269	-1863.9(486.6)	0.000	-6.80(46.99)	0.885	5.78(7.68)	0.454	-229.4(205.4)	0.268
Anti-TT IgG AI	0.00(0.00)	0.889	-0.25(0.19)	0.196	-0.01(0.02)	0.754	0.00(0.00)	0.687	-0.06(0.08)	0.475
Anti-DT IgG AI	-0.00(0.00)	0.806	-0.30(0.09)	0.001	0.00(0.01)	0.683	-0.00(0.00)	0.181	-0.03(0.04)	0.397
Anti-Measles IgG AI	0.00(0.00)	0.581	-0.14(0.07)	0.048	0.00(0.01)	0.983	0.00(0.00)	0.126	-0.03(0.03)	0.325
Anti-PPS IgG AI	-0.00(0.00)	0.028	0.14(0.12)	0.240	0.00(0.01)	0.916	-0.00(0.00)	0.190	0.08(0.05)	0.122
Anti-TT IgG ASC/1MPBMC	-0.02(0.08)	0.759	-3.33(11.87)	0.780	1.68(1.14)	0.148	0.23(0.19)	0.240	1.02(5.04)	0.841
Anti-Measles IgG	-0.16(0.11)	0.138	27.54(22.33)	0.225	-0.76(2.17)	0.728	-0.44(0.31)	0.162	20.16(9.02)	0.032
Anti-PPS IgG ASC/1MPBMC	-0.04(0.14)	0.745	4.79(24.94)	0.848	1.79(2.71)	0.513	0.11(0.40)	0.780	6.13(10.59)	0.565

Table 4.6: Estimated change in various B-cell responses in relation to variations in age, HIV status, viral loads, $CD4^+$ T-cell percentage and HAART treatment (beta coefficients). Every B-cell response was independently run in a generalized quantile regression model containing age, HIV status, viral load, $CD4^+$ T-cell percentage and HAART treatment. P values <0.05 were considered significant. TT-tetanus toxoid; DT-diphtheria toxoid; PPS-pneumococcal capsular polysaccharides; AI-avidity index; ASC/1MPBMCs-antibody secreting cells per 1 million PBMCs. Significant results are in red text.

4.3.4 Correlations between plasma antibody concentrations, antibody avidity indices and frequencies of IgG memory B cells.

Among the community controls, significant direct correlations were observed with regard to plasma IgG and avidity indices against TT, DT and measles. Similar correlations between plasma IgG and avidity indices were observed among HIV-infected children with regard to TT and DT only. There were also direct correlations between avidity indices and frequencies of memory B cells against TT and measles antigen among the HIV-infected children, but similar trends were not seen among the community controls. Interestingly, there was a direct correlation between plasma IgG and frequencies of memory B cells against pneumococcal capsular polysaccharides among the community controls.

	Community controls		HIV-infected	
	Plasma IgG	AI	Plasma IgG	AI
TT				
AI	0.8298 (<0.00005)		0.3887 (0.0053)	
ASC/1M PBMC	0.3792 (0.1207)	-0.0333 (0.8955)	0.4620 (0.0011)	0.4642 (0.0015)
DT				
AI	0.7214 (0.0007)		0.3804 (0.0038)	
Measles				
AI	0.3779 (0.0105)		0.0537 (0.5771)	
ASC/1M PBMC	0.3835 (0.1426)	0.1003 (0.6921)	0.0537 (0.7861)	0.5032 (0.0054)
PPS				
AI	-0.0326 (0.8398)		0.1034 (0.4085)	
ASC/1M PBMC	0.7608 (0.0065)	-0.0445 (0.8654)	0.2625 (0.0931)	0.1211 (0.4281)

Table 4.7: Spearman's correlation coefficients rho (P values) between plasma IgG levels, IgG avidity indices and frequencies of memory B cells against various antigens. The HIV-infected children and community controls have been evaluated separately. P values <0.05 were considered significant. TT-tetanus toxoid; DT-diphtheria toxoid; PPS-pneumococcal capsular polysaccharides; AI-avidity index; ASC/1MPBMCs-antibody secreting cells per 1 million PBMCs. Significant results are in red text.

4.4 Discussion

Similar to previous studies in children, in our study, HIV infection was associated with lower IgG levels against the childhood vaccine antigens tetanus toxoid, diphtheria toxoid and measles antigen[232, 245, 257-263]. In addition, HIV infection was associated with low avidity indices to TT, DT and measles antigens, confirming the results of a study that showed that HIV-infected children had lower avidity maturation of anti-measles IgG [261]. HIV infection was also associated with low frequencies of memory B cells against measles antigen, replicating the outcome in a study that showed that HIV-infected children who were started on HAART late had low memory B cell responses to measles regardless of subsequent control of viraemia [232]. Interestingly, HIV infection did not affect frequencies of anti-TT memory B cells, probably because of the small sample size that was left after omitting the children who had received the pneumococcal conjugate vaccine in the community. Furthermore, HIV infection had no effect on any anti-pneumococcal response, suggesting that it was not a good predictor of B-cell responses against environmental antigens. This could be due to an interaction between pneumococcal exposure and HIV infection. HIV-infected children could be exposed to more pneumococcal infections due to their impaired immune function or due to presence of sick HIV-infected parents in the household. As a result, the higher frequency of pneumococcal exposure could boost their anti-pneumococcal responses, making them comparable with HIV-uninfected children. Alternatively, HIV could be having no effect on B-cell responses against pneumococcal capsular polysaccharides. From the results, it was concluded that HIV-infection is associated with low antibody levels, poor avidity maturation and low frequencies of memory B cells to some of the childhood vaccines. Unlike most older studies, the current study had the strength of evaluating different types of B-cell responses (plasma IgG & IgM levels, IgG avidity indices and frequencies of memory B cells) against a variety of antigens that differed in

nature (i.e. subunit vaccines [toxoids], whole virus lysates [measles antigen] and polysaccharides) on the same children. Studies in adults have shown similar results with the exception of antibody avidity maturation that is relatively left intact in adults who get HIV-infected later in life after they have already been exposed to most antigens [84, 229, 243-245]. This is probably because antibody avidity maturation is a historical event that is relatively irreversible. As a result, adults would retain the antibody-avidity that they acquire prior to infection with HIV. On the other hand, children become infected with HIV in the first months of life and are exposed to most vaccines and environmental antigens when they are already HIV-infected. Their primary response (and any subsequent secondary responses) to most antigens would therefore take place in the presence of the HIV infection, leading to poor antibody avidity maturation.

Of note is the similarity between the various HIV-infected groups with regard to all measures of B-cell function, suggesting that control of viraemia (and the resultant recovery of $CD4^+$ T-cell counts) is not sufficient to restore the functionality of the compartment. Furthermore, in the multivariate analyses, viral load and $CD4^+$ T-cell percentages did not significantly predict any of the B-cell responses to vaccines, suggesting that the virological and immunological statuses at the chronic phase have little effect on the B-cell responses to historically encountered antigens. Interestingly, HAART treatment was a significant predictor of anti-measles memory B cells. In the univariate analysis, the HAART-treated children who had low viraemia showed higher frequencies of anti-measles and anti-pneumococcal memory B cells when compared with low viraemia HAART-naïve children, suggesting that initiation of HAART can influence at least some B cell responses. In agreement, another study showed that initiation of HAART in children in the first year of life helps to maintain the integrity of the memory B-cell compartment [232]. Furthermore, revaccination of successfully treated children and adults has been shown to elicit adequate responses [257, 264-268].

$CD4^+$ T-cell depletion provides a possible and straightforward explanation to the failure of HIV-infected patients to induce optimal B-cell responses. Since the quality and magnitude of a B-cell response is very dependent on the germinal centre reaction where $CD4^+$ T-cell help is crucial, depletion of $CD4^+$ T cells would arguably reduce the amount of T-cell help, resulting into a low quality and low magnitude of B-cell response. However, evidence suggests that the perturbation of the B-T cell interactions in HIV goes beyond mere depletion of helper T cells. For instance, B cells from viraemic HIV-infected people have been shown to respond poorly to $CD4^+$ T-cell help when compared with B cells from healthy uninfected and aviraemic HIV-infected people [247]. Such B cells are also inefficient in providing co-stimulation to $CD4^+$ T cells, suggesting that the $CD4^+$ T cells in viraemic patients might be poorly programmed to in turn give T-cell help [273]. Indeed, a more recent study showed that follicular helper T cells that are exposed to B cells from viraemic HIV patients are defective in performing their helper functions. The defects were shown to be a result of enhanced PD1-PD-L1 interaction that renders the follicular helper T cells incapable of producing adequate IL21 among other helper signals [274].

Alternatively, some HIV factors could directly affect the quality and magnitude of B-cell response. One such factor is HIV Nef that has been shown to impair class switching of naïve B cells in vitro by inhibiting CD40L dependent intracellular signaling via NFκB pathway. Considering that NFκB plays a central role in many other B-cell pathways, HIV Nef could therefore also affect other aspects of the B-cell response [25].

The mechanism for depletion of pre-existing antibody and memory B-cell responses to non-HIV antigens in HIV infection is poorly understood. Increased susceptibility of B cells to apoptosis has been reported and associated with increased expression of Fas in both chronic and primary HIV viraemic states [248-250]. The increased Fas mediated apoptosis of B cells has been attributed to a concerted effect of lymphopenia, viraemia

and T-cell activation [251]. Altered expression of receptors to BAFF on some B-cell subsets in HIV patients has also been implicated in permitting the Fas mediated apoptosis [275]. In addition, Foxo3a- and TRAIL- mediated apoptosis has been reported, suggesting that multiple pathways could contribute to the increased susceptibility to apoptosis that is seen in B cells from HIV-infected patients [276].

Finally, in this study, there was a notable direct correlation between the three measures of B-cell function in some of the groups of the children for some of the antigens. For instance, significant direct correlations were observed between all measures of anti-TT responses among the HIV-infected children. This could be an indication of the efficiency of the immune response that each child had experienced at the time of immunisation; a good responder is likely to generate more long-lived plasma cells and memory B cells while experiencing more antibody avidity maturation. On the other hand, the correlation could be an indicator of the dependency on the memory B-cell pool for the maintenance of the plasma-cell pool, as suggested by Bernasconi and colleagues [277]. However, the reason for absence of such correlations of anti-TT responses among the community controls is not clear. Notably, there were no correlations between various responses against pneumococcal capsular polysaccharides, probably due to the complex interactions between exposure, HIV disease progression and the complex biological properties of pneumococcal bacteria.

Chapter 5 Memory B-cell responses in healthy children who received a 10-valent Pneumococcal non-typeable *Haemophilus Influenza* protein D conjugate vaccine (PHiD-CV)

5.1 Literature review

Streptococcus pneumoniae is a major cause of morbidity and mortality in the world, accounting for an estimated 14.5 million cases of serious illness and 735000 deaths in HIV-negative children below five years of age in the year 2000 [278]. In Kenya, it has been shown to be the leading cause of invasive bacterial disease in children [279, 280]. Introduction of pneumococcal conjugate vaccine in some countries' childhood immunization schedules has led to a dramatic reduction of the incidence of Invasive Pneumococcal Disease (IPD) among the vaccinated children [281]. It has also had an indirect effect of reducing pneumococcal disease in the general population, probably by reducing transmission of disease from vaccinated children to unvaccinated children and adults [282, 283].

Synflorix^{tm} is a 10-valent Pneumococcal non-typeable *Haemophilus Influenza* protein D conjugate vaccine (PHiD-CV). It contains ten pneumococcal capsular polysaccharides that are conjugated to protein D (for serotypes 1, 4, 5, 6B, 7F, 9V, 14 and 23F), tetanus toxoid (for serotype 18C) and diphtheria toxoid (for serotype 19F). The protein D is derived from a cell-surface lipoprotein that is conserved among both capsulated and unencapsulated strains of *Haemophilus influenzae.* PHiD-CV targets additional serotypes 1, 5 and 7F that are not targeted by the 7-valent pneumococcal conjugate vaccine (PCV7) that has been used in many developed countries since 2001, but has now been replaced with a 13-valent version. These three serotypes are important causes of IPD in developing countries [284, 285]. In general, the 10 serotypes targeted by PHiD-CV are responsible for approximately 70% of IPD in Kenyan children below the age of five years [286].

Studies in Europe, South America and Asia have shown that PHiD-CV is comparably immunogenic when compared with PCV7 and when co-administered with other

childhood vaccinations [287-290], leading to its licensure in more than 100 countries for use in children below 2 years of age. Based on recent data, the age limit for the administration of the vaccine has been extended to 5 years in the European Union [291]. These immunogenicity studies have been done by measuring serum IgG (by ELISA) and opsonophagocytic activity (OPA). Notably, the antibody titres and opsonophagocytic activity after vaccination wane over time but increase markedly after booster vaccination, suggesting that priming after the primary vaccination induces immunological memory. However, direct evaluation of the generation of serotype-specific memory B cells after administration of PHiD-CV needs to be done.

In this study, I determined the generation of memory B cells against pneumococcal capsular polysaccharides and the conjugating proteins (tetanus toxoid and diphtheria toxoid) after administration of PHiD-CV among community children aged 12-24 months. The study was part of a larger clinical trial that evaluated the immunogenicity and reactogenicity of the vaccine among children aged 12-59 months. Due to the limited amount of sample that could be obtained from the children, the assays were limited to serotypes 1, 6B, 14, 19F and 23F. These serotypes were chosen based on available data on their contribution to IPD and nasopharyngeal carriage. A previous study had shown the rate of isolation of various vaccine type serotypes from children presenting with IPD in Kilifi district to rank as follows: serotypes 1>14>6B>6A>5>23F>4 [286]. Another study on nasopharyngeal pneumococcal carriage showed that the rate of serotype-specific carriage in Kilifi district ranked as follows: serotypes 19F>6A>6B>23F>14>9V>7C>1>4 [292].

Determination of memory B cells at day 0 informed on the effect of pre-existing naturally acquired memory B-cell responses on the response to PHiD-CV while determination of frequencies of memory B cells at day 30 (30 days after first dose of

PHiD-CV) and at day 210 (30 days after the second dose of PHiD-CV) informed on the ability of the PHiD-CV to elicit memory B cells against the various antigens. Determination of pneumococcal carriage on day 0 was important in determining the effect of nasopharyngeal carriage on responses to PHiD-CV while determination of carriage on day 180 was done to determine if responses elicited after a single dose of PHiD-CV affected subsequent nasopharyngeal carriage.

The frequencies of antigen-specific memory B cells in the children in this study were then compared with those of HIV-infected children (CCRC) and community controls (Ngerenya) that had either received or not received the PHiD-CV vaccine (some of the children were reported in chapter 4). Of particular importance was the comparison between the PHiD-CV vaccinated children before they received the PHiD-CV vaccine and the HIV-infected children from CCRC who did not receive the PHiD-CV vaccine. Notably, the PHiD-CV vaccinated children were younger than the children from CCRC and community controls from Ngerenya. Nevertheless, a comparison between older HIV-infected children and younger community children would be worthwhile especially if HIV-infection causes immunological stunting with regard to natural acquisition of immunity against environmental pathogens like *Streptococcus pneumoniae.* Indeed, HIV has been shown to interfere with the age-related natural acquisition of anti-malarial responses in children [293].

5.2 Objectives

1. To determine the frequencies of memory B cells against various antigens before PHiD-CV vaccination and at follow-up among healthy children.

2. To determine the relationship between frequencies of memory B cells and plasma antibodies against the pneumococcal capsular polysaccharides
3. To determine the relationship between nasopharyngeal carriage and frequencies of memory B cells with regard to the various serotypes.
4. To compare frequencies of memory B cells in the healthy children in the PRISM study with HIV-infected children from CCRC and healthy children from Ngerenya.

5.3 Results

5.3.1 Population characteristics

600 children were recruited into the PHiD-CV randomized clinical trial, 200 into each arm of the study. PBMC samples collected from 35 children in group B (those who received the PHiD-CV vaccine at enrollment and on day 180 as per table 2.1) were used for determination of memory B cells against pneumococcal capsular polysaccharides, tetanus toxoid and diphtheria toxoid. They were selected based on their age (12-24 months) in order to limit the sample size due to the laborious nature of the assays. Data on pneumococcal nasopharyngeal carriage and plasma antibody levels were obtained from the principal investigator of the PHiD-CV randomized clinical trial. The population characteristics of the 35 children are shown in table 5.1.

Characteristic	Value
Total number	35
Median age (IQR) in months	20 (16-22)
Females (%)	19(54%)
Median Children in household (range)	3(1-6)
Smokers in household (%)	11(31%)
Had 3 doses of Pentavalent vaccine (DTaP+HB+HiB)	91.4%
Had at least 1 dose of Pentavalent vaccine	100.0%

Table 5.1: Population characteristics of the children who participated in the memory B cells substudy.

IQR-Interquartile range.

Among the 35 children who participated in the memory B cell substudy, there was a decline in total carriage when baseline pneumococcal carriage was compared with pneumococcal carriage at day 180 (From 88.6% to 71.4%). There was also a marked reduction in carriage of vaccine-type serotypes (including serotype 6A) when the two time points were considered (From 51.4% to 35.3%). The carriage rate of non-vaccine serotypes remained unchanged at 37.1%.

When the various pneumococcal serotypes were considered, there was a decline in carriage for serotypes 19F and 23F amongst the sub-study's participants when day zero (0) was compared with day 180. Carriage of serotype 6B remained unchanged while that of serotypes 14 and 1 remained low at the two time points. Carriage of individual non-vaccine serotypes was low, with all being carried by less than 6% of the children at the two time points.

Serotype	Baseline Number (%)	Day 180 Number (%)
10A	1(2.9%)	0(0.0%)
11A	1(2.9%)	1(2.9%)
13	1(2.9%)	0(0.0%)
14	1(2.9%)	1(2.9%)
15A	1(2.9%)	2(5.7%)
15B	1(2.9%)	0(0.0%)
15C	1(2.9%)	2(5.7%)
16A	1(2.9%)	0(0.0%)
16F	1(2.9%)	1(2.9%)
19A	1(2.9%)	0(0.0%)
19B	1(2.9%)	0(0.0%)
19F	6(17.1%)	1(2.9%)
20	0(0.0%)	1(2.9%)
21	1(2.9%)	2(5.7%)
23B	1(2.9%)	1(2.9%)
23F	4(11.4%)	1(2.9%)
31	1(2.9%)	0(0.0%)
34	0 (0.0%)	2(5.7%)
35B	0(0.0%)	1(2.9%)
6A	3(8.6%)	3(8.6%)
6B	4(11.4%)	4(11.4%)
9V	0(0.0%)	2(5.7%)
1	0(0.0%)	0(0.0%)
18C	0(0.0%)	0(0.0%)
7F	0(0.0%)	0(0.0%)
4	0(0.0%)	0(0.0%)
5	0(0.0%)	0(0.0%)
Total	**31(88.6%)**	**25(71.4%)**
Vaccine serotypes	**18(51.4%)**	**12(35.3%)**
Non vaccine type	**13(37.1%)**	**13(37.1%)**

Table 5.2: Frequencies of nasopharyngeal carriage of various pneumococcal serotypes at baseline and at day 180 of the study. Vaccine serotypes have been shown in red text. Serotype 6A has been included among the vaccine serotypes because of the antigenic cross-reactivity between it and serotype 6B.

When the IgG levels against the 5 polysaccharides that were evaluated in the memory B cells sub-study were considered, there was a significant increase in IgG concentrations against capsular polysaccharides for all five serotypes after each vaccination with 91-100% of the children attaining at least 0.26 μg/ml of anti-capsular polysaccharide IgG against the various pneumococcal capsular polysaccharides after the second dose.

Serotype	Time point			P values	
	Day 0	Day 30	Day 210	Day 30 vs Day 0	Day 210 vs Day 0
% of responders					
19F	23%	80%	94%		
14	29%	86%	100%		
6B	34%	74%	97%		
23F	11%	63%	91%		
1	9%	100%	97%		
Median (IQR) IgG levels					
19F	0.11 (0.06-0.24)	3.22 (0.47-10.82)	19.50 (2.70-33.50)	<0.0001	<0.0001
14	0.02 (0.022-0.39)	1.05 (0.47-2.46)	4.80 (2.21-11.75)	<0.0001	<0.0001
6B	0.21 (0.10-0.38)	0.60 (0.25-1.15)	1.57 (0.86-3.57)	<0.0001	<0.0001
23F	0.03 (0.00-0.09)	0.50 (0.13-0.94)	1.02 (0.44-1.83)	<0.0001	<0.0001
1	0.08 (0.04-0.15)	1.06 (0.48-2.12)	2.05 (1.43-4.40)	<0.0001	<0.0001

Table 5.3: IgG responses to various pneumococcal capsular polysaccharides. Wilcoxon signed-rank test was used to compare levels of antibodies at different time points.

5.3.2 Frequencies of antigen-specific memory B cells

Serotype 19F exemplified the highest baseline and vaccine induced frequencies of memory B cells. After the first dose of PHiD-CV, there was a significant increase in the

frequency of memory B cells against serotype 19F (From 0 [IQR, 0-4] ASC/1M PBMCs to 15 [IQR, 0-28] ASC/1M PBMCs, P=0.0046). Similarly, there was a significant increase in frequencies of memory B cells against serotype 19F after the second dose of PHiD-CV (From 15 [IQR, 0-27.5] ASC/1M PBMCs to 22 [IQR, 0-61] ASC/1M PBMCs, P=0.0275). For serotype 14, the frequencies of memory B cells did not significantly change after the first dose of PHiD-CV (From 0 [IQR, 0-3] ASC/1M PBMCs to 0 [IQR, 0-8] ASC/1M PBMCs, P=0.1242), but did increase significantly after the second dose of PHiD-CV (From 0 [IQR, 0-8] ASC/1M PBMCs to 5 [IQR, 0-21] ASC/1M PBMCs, P=0.0294). Notably, there was no significant increase in the frequencies of anti-PS6B memory B cells after the first (From 0 [IQR, 0-5] ASC/1M PBMCs to 3 [IQR, 0-6] ASC/1M PBMCs, P=0.2596) and second (From 3 [IQR, 0-6] ASC/1M PBMCs to 2.5[IQR, 0-12] ASC/1M PBMCs, P=0.0867) doses of PHiD-CV. Also, the frequencies of anti-PS6B memory B-cell after the two doses of PHiD-CV did not differ from baseline frequencies (P=0.2417). On the other hand, while there was no significant increase of anti-PS23F memory B cells after the first (From 0 [IQR, 0-3] ASC/1M PBMCs to 0 [IQR, 0-4] ASC/1M PBMCs, P=0.3027) and the second (From 0 [IQR, 0-4] ASC/1M PBMCs to 2.5 [IQR, 0-14] ASC/1M PBMCs, P=0.1269) doses of PHiD-CV, there was a significant difference when frequencies of memory B cells after the two doses were compared with baseline frequencies (P=0.0068). Anti-PS1 memory B–cell frequencies were higher after the first (5 [IQR, 0-10] ASC/1M PBMCs, P=0.0005) and second (4 [IQR, 0-15] ASC/1M PBMCs, P=0.0006) doses of PHiD-CV when compared with baseline (0 [IQR, 0-0] ASC/1M PBMCs). However, there was no difference between frequencies at day 30 and day 180 (P=0.1024), implying that a single dose of PHiD-CV had an effect that was similar to two doses with regard to this serotype.

In this vaccine, all capsular polysaccharides were conjugated to protein D from non-typeable *Haemophilus Influenza,* with the exception of serotypes 18C and 19F that were conjugated to tetanus toxoid and diphtheria toxoid respectively. Furthermore, the children in this sub-study received a booster dose of DTaP on day 60. Almost all of them had received three doses of the pentavalent (DTaP+HB+HiB) immunization in their first year of life. The PHiD-CV vaccination had not been introduced into the childhood vaccination schedule at the time when these children were receiving the pentavalent immunization. We therefore aimed to also determine the effect of the study on the frequencies of memory B cells to tetanus toxoid and diphtheria toxoid. After the first dose of PHiD-CV, there was a significant increase in frequencies of memory B cells against tetanus toxoid (From 20 [IQR, 5-33] ASC/1M PBMCs to 45 [IQR, 10-119] ASC/1M PBMCs, P=0.0105) and diphtheria toxoid (From 11 [IQR, 0-33] ASC/1M PBMCs to 65 [IQR, 35-115] ASC/1M PBMCs, P=0.0087). However, there was no further increase following a booster dose of DTaP on day 60 and a second dose of PHiD-CV on day 180 with regard to both proteins (Tetanus toxoid: from 45 [IQR, 10-119] ASC/1M PBMCs to 58 [IQR, 24-127] ASC/1M PBMCs, P=0.6494; Diphtheria toxoid: from 65 [IQR, 35-115] ASC/1M PBMCs) to 66 [IQR, 38-166] ASC/1M PBMCs, P=0.2238).

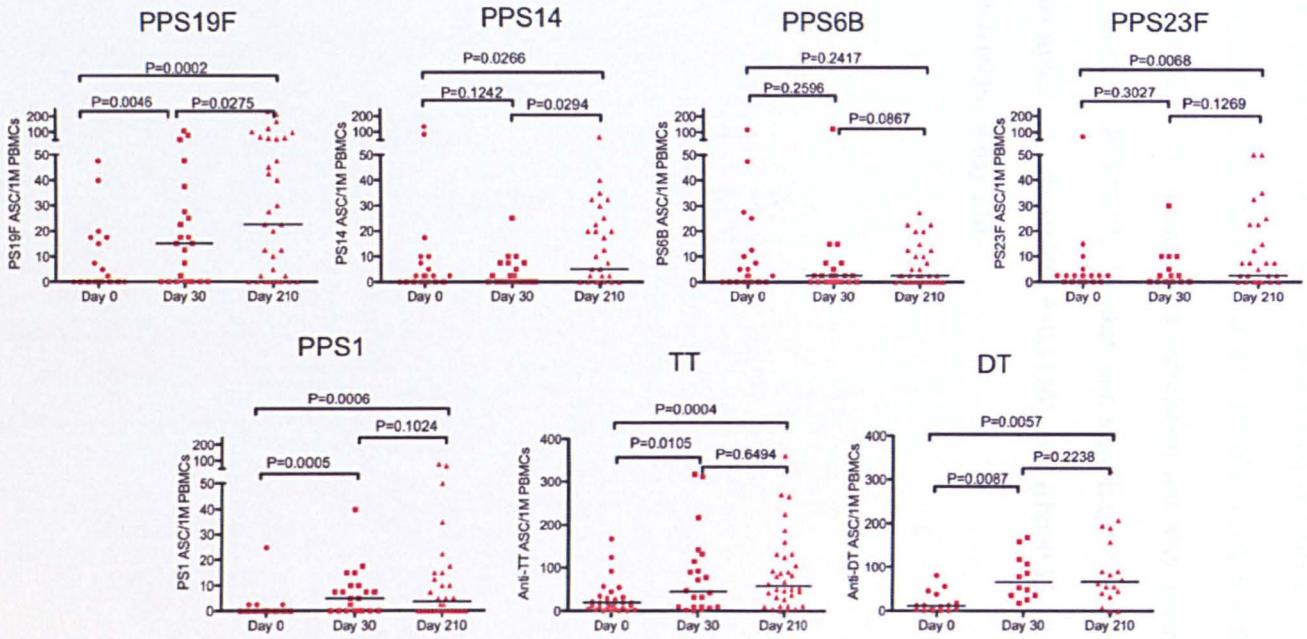

Figure 5.1: Frequencies of antigen specific memory B cells against various pneumococcal capsular polysaccharides (PS), tetanus toxoid (TT) and diphtheria toxoid (DT). The vaccine induced significant increase in frequencies of memory B cells against all constituent antigens tested with the exception of serotype 6B. Horizontal line represents median. Statistical test used: Wilcoxon signed-rank test.

5.3.3 Correlation between anti-pneumococcal plasma IgG and frequencies of anti-pneumococcal memory B cells

There was no correlation between plasma IgG levels and frequencies of memory B cells at baseline for the various capsular polysaccharides. However, a direct correlation emerged for serotype 19F at day 30 (rho=0.55, P=0.0069) and day 210 (rho=0.68, P<0.00005). A similar direct correlation was also observed for serotype 1 at day 210 (rho=0.55, P=0.0019). Weaker non-significant direct correlations were observed for serotypes 14 (rho=0.30, P=0.1116), 6B (rho=0.35, P=0.0545) and 23F (rho=0.30, P=0.0936) at day 210.

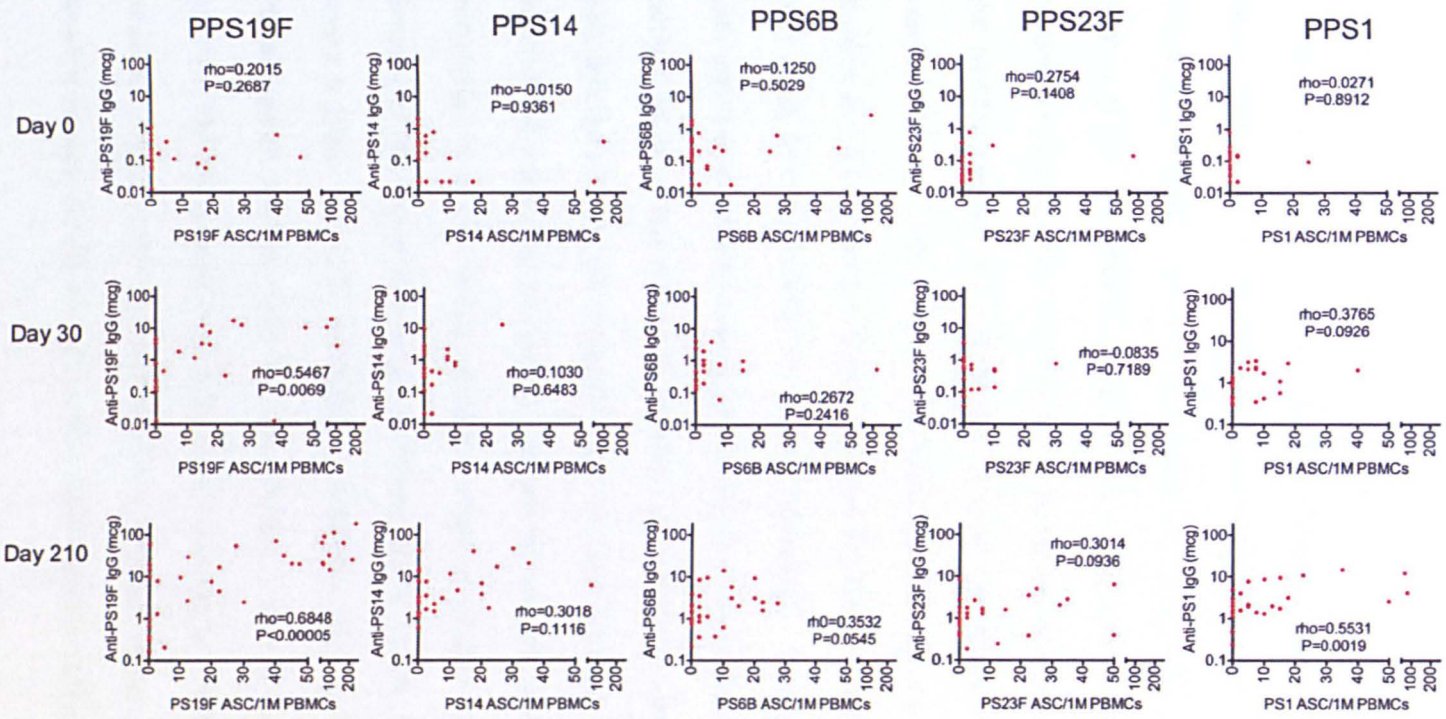

Figure 5.2: Correlation between plasma antibodies and frequencies of memory B cells against the antigens at different time points. There was significant correlation of anti-PS19F antibodies and memory B cells at days 30 and 210 and anti-PS1 antibodies and memory B cells at day 210. Similar but weaker non-significant correlations were also seen for the other antigens at day 210. Statistical test: Spearman's correlations.

5.3.4 Effect of baseline pneumococcal carriage on the frequencies of anti-pneumococcal memory B cells

When the children were classified on the basis of their baseline nasopharyngeal carriage for the various serotypes, the six children that had serotype 19F carriage at day 0 also had significantly lower frequencies of anti-PS19F memory B cells at days 30 (0 [IQR, 0-0] ASC/1M PBMCs versus 18 [IQR, 10-43] ASC/1M PBMCs, P=0.0013) and 210 (0 [IQR, 0-9] ASC/1M PBMCs versus 35 [IQR, 4-66] ASC/1M PBMCs, P=0.0114) when compared with children who did not have carriage for serotype 19F at day 0, suggesting that baseline carriage was associated with lower frequencies of memory B-cell to the vaccine. However, there was no difference between the two groups with regard to baseline anti-PS19F memory B-cell responses (0 [IQR, 0-9] ASC/1M PBMCs versus 0 [IQR, 0-5] ASC/1M PBMCs, P=0.6946). Interestingly, the same trend was observed with regard to anti-PS19F plasma IgG levels whereby children who had serotype 19F carriage on day 0 had lower IgG responses against PS19F when they were compared with children who had no carriage on day 0. On the other hand, there was no difference in anti-PS6B memory B-cell response between the serotype 6B/6A baseline carriers and non-carriers at all time points; baseline carriage of serotype 6B/6A did not affect the frequencies of memory B cells against PS6B (Day 0, 0 [IQR, 0-48] ASC/1M PBMCs versus 0 [IQR, 0-5] ASC/1M PBMCs, P=0.9570; Day 30, 3 [IQR, 0-5] ASC/1M PBMCs versus 3 [IQR, 0-42] ASC/1M PBMCs, P=0.4351; Day 210, 3 [IQR, 0-11] ASC/1M PBMCs versus 3 [IQR, 0-20] ASC/1M PBMCs, P=0.8505). The frequency of baseline carriage of the other serotypes was very low in our cohort and comparison between baseline carriers and non-carriers could therefore not be done.

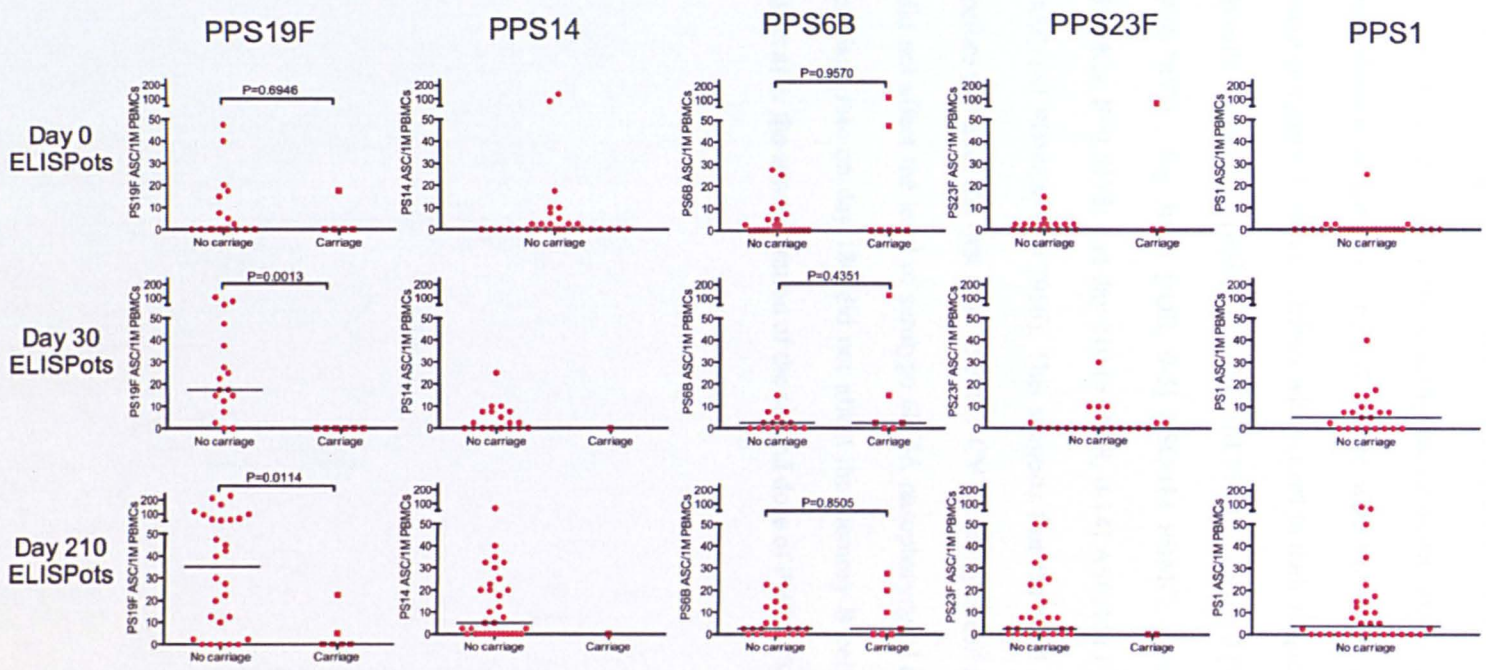

Figure 5.3: Frequencies of antigen specific memory B cells against the various pneumococcal capsular polysaccharides (PS) after stratifying the study participants on the basis of their baseline pneumococcal carriage of the respective serotypes. Baseline carriage of serotype 19F was associated with weaker subsequent anti-PS19F memory B-cell responses. Anti-PS6B memory B-cell responses did not differ between baseline serotype 6B carriers and non-carriers. Horizontal line represents median. Statistical test used: Wilcoxon rank-sum test.

On day 180, there was little or no nasopharyngeal carriage for serotypes 19F, 14, 23F and 1. Consequently, the effect of the memory B-cell response on level of carriage could not be assessed for these serotypes. However, for serotypes 6B/6A whose day 180 nasopharyngeal carriage was 20%, there were no differences when day 180 carriers were compared with non-carriers with regard to their frequencies of anti-PS6B memory B cells for day 0 (0 [IQR, 0-5] ASC/1M PBMCs versus 0 [IQR, 0-8] ASC/1M PBMCs, P=0.7877) , day 30 (3 [IQR, 0-5] ASC/1M PBMCs versus 3 [IQR, 0-36] ASC/1M PBMCs, P=0.5378) and day 210 (3 [IQR, 0-14] ASC/1M PBMCs versus 3 [IQR, 0-10] ASC/1M PBMCs, P=0.7956). This suggests that the level of memory B-cell response before and after the first dose of PHiD-CV (memory B cell responses on days 0 and 30) did not affect the level of serotype 6B/6A nasopharyngeal carriage on day 180 and the carriage rate on day 180 did not affect the memory B cell responses on day 210 (30 days after the administration of the second dose of PHiD-CV on day 180).

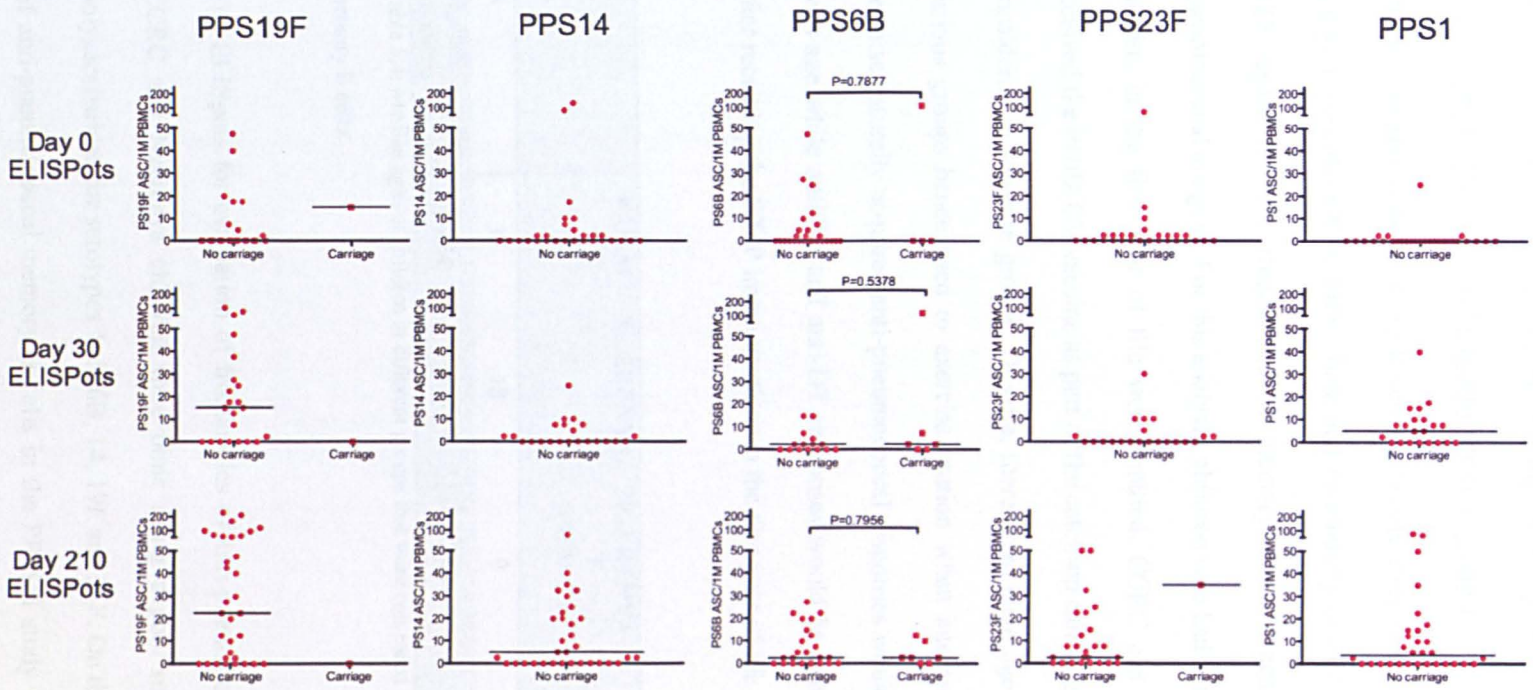

Figure 5.4: Frequencies of antigen specific memory B cells against the various pneumococcal capsular polysaccharides (PS) after stratifying the study participants on the basis of their day 180-pneumococcal carriage of the respective serotypes. Anti-PS6B memory B-cell responses did not differ between day 180 serotype 6B carriers and non-carriers. Horizontal line represents median. Statistical test used: Wilcoxon rank-sum test.

5.3.5 Comparison of healthy children in the PRISM study with HIV-infected children from CCRC and healthy children from Ngerenya

The 35 children in the memory B cells sub-study were then compared to HIV-infected children from the CCRC HIV clinic and community control children from Ngerenya with regard to their frequencies of memory B cells against tetanus toxoid and pneumococcal antigens. For this analysis, children who had not received the pentavalent vaccine in the first year of life were omitted. CCRC and Ngerenya children who received the PHiD-CV vaccine as part of the catch-up campaign in the community were considered in separate groups. Of note, there were huge age differences between the various groups hence need to exercise caution when interpreting the results. This is because naturally acquired anti-pneumococcal responses would be expected to increase with age while anti-TT and anti-DT responses would be expected to wane over time after receiving the DTaP immunizations in the first year of life.

	PRISM	**NGERENYA**	**NGERENYA + PHiD-CV**	**HIV**	**HIV + PHiD-CV**
N	35	18	9	46	4
Median age (months)	12-24	78.5	33.7	48.5	21

Table 5.4: Median ages of children in different groups that were compared with regard to frequencies of memory B cells.

The ELISpots for evaluation of frequencies of anti-pneumococcal memory B cells in CCRC and Ngerenya children were done using a pool of pneumococcal capsular polysaccharides for serotypes 1, 5, 6B, 14, 19F and 23F. On the other hand, frequencies of anti-pneumococcal memory B cells in the PRISM study were done in a serotype-specific manner for serotypes 1, 6B, 14, 19F and 23F. Therefore, evaluation for serotype 5 was only done in the CCRC and Ngerenya children. As a result, comparisons

of the different groups of children were done with the awareness that responses against serotype 5 had been done for some groups only. Frequencies of memory B cells against the various pneumococcal capsular polysaccharides in the PRISM children were summed up and compared with frequencies of memory B cells against the pool of polysaccharides that was used for the CCRC and Ngerenya children.

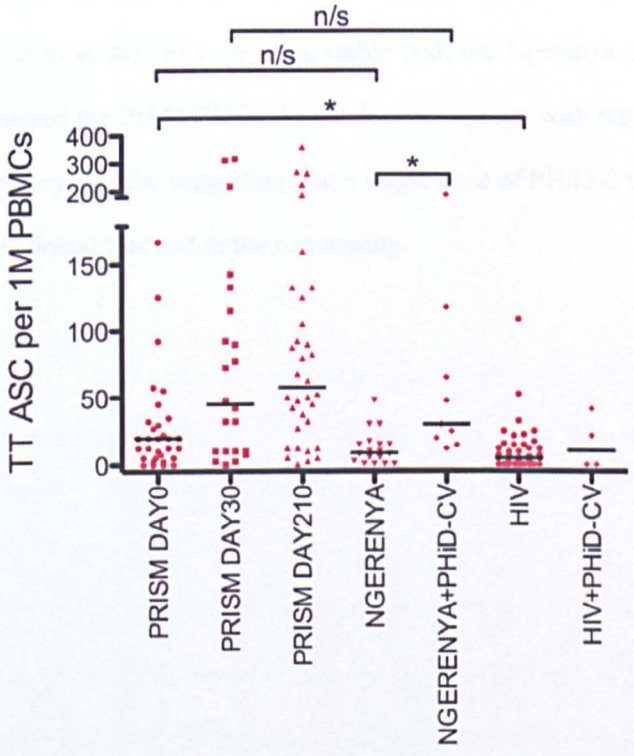

Figure 5.5: Comparison of PRISM children with CCRC HIV-infected children and community controls from Ngerenya with regard to frequencies of memory B cells against tetanus toxoid. Horizontal lines show median. *P<0.05. n/s-no significant difference (i.e. P>0.05).

When the different children were compared with regard to frequencies of memory B cells against tetanus toxoid, the PRISM children at day 0 were comparable with the Ngerenya community controls who had not received the PHiD-CV vaccine. However,

the PRISM children at day 0 had higher frequencies of memory B cells against TT when they were compared with HIV-infected children who had not received the PHiD-CV vaccine, suggesting that the HIV-infected children had experienced faster waning of anti-TT memory B cells when compared with the Ngerenya healthy children. As would be expected, the Ngerenya community controls who had received the PHiD-CV had higher frequencies of anti-TT memory B cells when compared with those who had not received the PHiD-CV, suggesting that administration of the PHiD-CV vaccine in the community indeed boosted anti-TT and probably also anti-DT responses. The PRISM children at day 30 were comparable with the Ngerenya community controls who had received the PHiD-CV in the catch up campaign with regard to frequencies of anti-TT memory B cells, suggesting that a single dose of PHiD-CV elicited similar responses in the clinical trial and in the community.

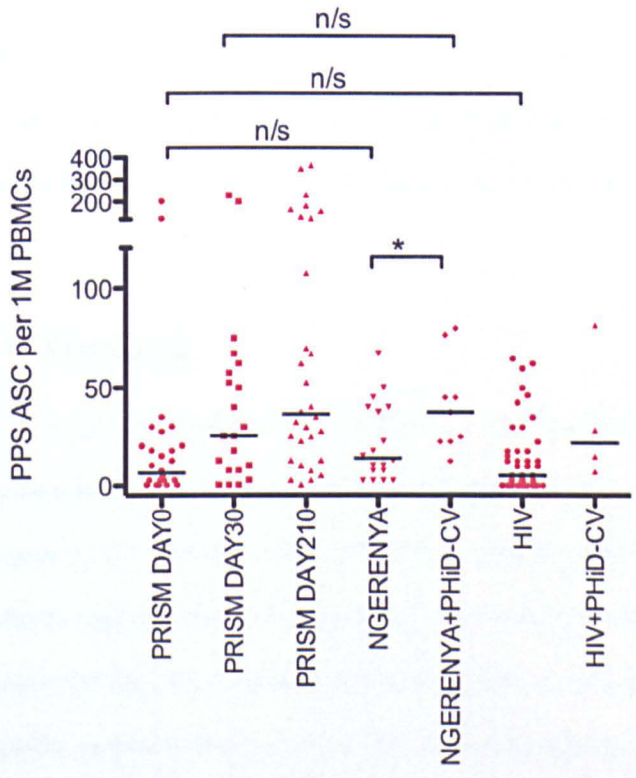

Figure 5.6: Comparison of PRISM children with CCRC HIV-infected children and community controls from Ngerenya with regard to frequencies of memory B cells against pneumococcal capsular polysaccharides. Horizontal lines show median. *P<0.05. n/s-no significant difference (i.e. P>0.05).

When the different children were compared with regard to frequencies of memory B cells against pneumococcal capsular polysaccharides, the PRISM children at day 0 were comparable with the Ngerenya community controls who had not received the PHiD-CV vaccine. Similarly, the PRISM children at day 0 were comparable with HIV-infected children who had not received the PHiD-CV vaccine with regard to frequencies of memory B cells against pneumococcal capsular polysaccharides. As would be expected, the Ngerenya community controls who had received the PHiD-CV had higher frequencies of anti-pneumococcal memory B cells when compared with those who had not received the PHiD-CV, suggesting that administration of the PHiD-CV vaccine in the community indeed elicited anti-pneumococcal memory B-cell responses. The

PRISM children at day 30 were comparable with the Ngerenya community controls who had received the PHiD-CV in the catch up campaign with regard to frequencies of anti-pneumococcal memory B cells, suggesting that a single dose of PHiD-CV elicited similar responses in the clinical trial and in the community.

5.4 Discussion

In our study, the induction of memory B-cell responses to the studied pneumococcal capsular polysaccharides differed between the antigens.

In general, the presence of some baseline responses against all serotypes in some of the children suggests that natural exposure can induce the formation of memory B cells against the capsular polysaccharides. Indeed, antibody responses against pneumococcal capsular polysaccharides increase upon natural exposure and are thought to contribute to the age-dependent naturally acquired immunity to pneumococcal disease [294].

Notably, the baseline memory B-cell responses against serotype 19F were much higher than those against any of the other four serotypes, suggesting that the children had encountered more natural exposure to serotype 19F prior to vaccination. Previous studies in Kenya have shown that there is more nasopharyngeal carriage of serotype 19F in the community when compared to carriage of the other four serotypes [292]. Another important observation was the fact that memory B-cell responses against serotype 19F after vaccination with PHiD-CV were stronger than those of any other serotype measured. This could be attributed to the high baseline responses due to higher previous exposure to PS19F. This could have primed the children against PS19F, hence making them to have higher vaccine-induced responses to PS19F when compared with other serotypes. On the other hand, the conjugation of PS19F to diphtheria toxoid, an antigen that the children had experienced during their childhood pentavalent (DTaP+HB+HiB) vaccination series, could have influenced the amount of T-cell help

available to PS19F specific B cells. This is because DTaP vaccination would be expected to have induced formation of helper T cells against diphtheria toxoid peptides that would be presented by PS19F specific B cells after the vaccination.

From our results, a single dose of the vaccine induced a significant increase in frequencies of anti-PS19F memory B cells while a second dose induced a further increase, suggesting that every successive dose had a beneficial effect on this compartment. The first dose was essentially a boost of the pre-existing high natural responses against PS19F. On the other hand, the first dose was ineffective in inducing anti-PS14 memory B cells. A second dose successfully induced an increase of anti-PS14 memory B cells; two doses would be required to have an effect on this compartment. The same effect was observed with regard to serotype 23F whereby a significant increase of anti-PS23F memory B cells was only detected after the second dose. For serotype 1, the first dose induced a significant increase in anti-PS1 memory B cells but the second dose was ineffective in inducing a further increase, suggesting that one dose was required whereas a subsequent dose conferred no additional benefit. Of note was the inability of the vaccine to induce anti-PS6B memory B cells, a fact that resonated with the inability to reduce nasopharyngeal carriage against serotypes 6B/6A in our cohort and the entire cohort of the larger vaccine trial.

Frequencies of memory B cells against tetanus toxoid and diphtheria toxoid also increased after the first dose. The vaccine can therefore confer an additional benefit of boosting B-cell responses against the DPT vaccine.

The direct correlation between the antibody levels and the frequencies of memory B cells against PS19F and PS1 at day 210 could be an indication of the strength of the children's germinal centre reaction to the antigens. Arguably, a child who makes a strong germinal centre reaction would be expected to concurrently induce higher antibody levels and more memory B cells when compared with a child who makes a

weaker germinal centre reaction, though differentiation into either memory B cells or plasma cells could also occur independently since they are driven by different factors. Similar correlations and explanations have been observed and discussed in chapter four with regard to B-cell responses against tetanus toxoid and measles antigen. Considering that similar but non-significant trends were observed for the other serotypes at day 210, it could be a universal phenomenon that was only missed out in some serotypes due to the limited number of our study subjects and the varying immunogenicity of the antigens. However, polysaccharide antigens have been reported to induce T-independent antibody responses that are independent of the germinal centre reaction. As a result, even though the capsular polysaccharides in the vaccine were conjugated to proteins to give them the ability to induce T-dependent responses, they could have retained their ability to also cross-link BCR and induce T-independent responses, leading to generation of antibodies with limited induction of memory B cells via that route. Such an effect could be an explanation to the poor correlation between levels of plasma IgG and frequencies of memory B cells.

Surprisingly, the children who had baseline nasopharyngeal carriage of serotype 19F also had lower anti-PS19F antibody and memory B-cell responses to the vaccine. Previously, nasal carriage has been shown to induce plasma antibody responses to some capsular polysaccharides [294]. In addition, pneumococcal nasal challenge without carriage has been shown to induce mucosal IgG against protein antigens but not against capsular polysaccharides, implying that priming and boosting of immune responses can occur in the absence of active carriage as long as an individual gets exposed to the bacteria in the community [295]. The natural exposure of our participants to pneumococcal challenge (and whether it resulted in carriage or no carriage) was not determined. It is possible that both carriers and non-carriers had experienced natural challenge, with the non-carriers eliciting better priming of systemic B-cell responses

while also making better mucosal responses that would prevent carriage. Such priming in the children without nasopharyngeal carriage would explain their better responses to the vaccine when compared with children who had baseline carriage.

Day 180 serotype 6B/A carriage was not associated with the level of anti-PS6B memory B-cell responses. This could be interpreted to mean that anti-PS6B B-cell responses did not protect against carriage, an argument that fits well with the fact that there was no reduction in carriage of serotype 6B in the larger vaccine trial despite the attainment of adequate anti-6B IgG levels.

The observation that Ngerenya community controls who received the PHiD-CV vaccine had higher anti-TT and anti-pneumococcal memory B-cell responses when compared with Ngerenya community controls who did not receive PHiD-CV proves that the vaccine was effective in inducing memory B cells when administered in the community. The similarity of the Ngerenya PHiD-CV vaccinated children with the PRISM children at day 30 (after 1 dose of PHiD-CV) implies that the effectiveness of PHiD-CV was similar in the clinical trial and in the catch-up campaign in the community. Interestingly, older HIV-infected children were comparable to younger healthy children in the absence of PHiD-CV, suggesting that HIV had impaired their ability to acquire anti-pneumococcal memory B cells naturally with age. Due to the small numbers of HIV-infected PHiD-CV vaccinated children, I was unable to evaluate the effectiveness of the vaccine amongst HIV-infected children.

In summary, I have shown that immunization with PHiD-CV elicits memory B-cell responses against pneumococcal capsular polysaccharides in the setting of a controlled clinical trial as well as in the community setting. I have also shown that the vaccine boosts anti-TT and anti-DT memory B-cell responses. The similarity of older HIV-infected children to younger healthy children with regard to naturally acquired anti-

pneumococcal responses suggests that HIV infection could be impairing the natural acquisition of memory B cells to pneumococcal antigens.

Chapter 6 Effect of different combinations of B-cell stimulants and the in vitro effect of recombinant HIV-1 Nef on B-cell responses

6.1 Literature review

Even though most pathogens are able to elicit/carry all four signals that activate B cells, i.e BCR Ligand (antigen), TLR ligand (e.g pathogen genetic material), CD40L and cytokines, some antigens may not elicit some of the signals. For instance, immunisation with a recombinant protein that is devoid of TLR ligation may elicit BCR signalling and T-cell help without TLR signalling, though adjuvant can be used to trigger innate signals in such scenarios. Also, T-independent antibody responses have been proposed to occur in the absence of T-cell help [296]. Furthermore, bystander activation of memory B cells with irrelevant specificities has been proposed as a means by which the body maintains long lasting antibody responses to historically encountered antigens, suggesting that activation of some B cells can occur in the absence of BCR signalling [277]. All these possibilities point towards the importance of understanding the effect of all permutations of combinations of different signals on B cells. There is also a need to understand how various pathogens manipulate these B-cell stimulatory signals, singly or in combinations, as a means of blunting the antibody responses against themselves.

Indeed, most viruses have evolved ways of modulating the immune system. HIV is one such virus that, in addition to depleting host's $CD4^+$ T cells, causes many other defects in the immune system so as to evade clearance. As a result, HIV-infection is associated with a wide range of defects in all lymphocyte compartments. Some of the defects are thought to be due to the depletion of $CD4^+$ T cells. The observed impairment of the function of follicular helper T cells could also play a role in causing defects in the B-cell compartment [274]. Other defects could be due to the direct effect of virion factors such as viral proteins and viral RNA.

HIV-1 Nef is an accessory protein that has been linked to several immune-cell defects in HIV-infection. It is a 27-kDa protein that is expressed early in the replication

cycle of the virus [297]. Originally named 'negative factor', it was thought to negatively regulate viral replication [298]. However, subsequent studies showed its importance in HIV-1 pathogenesis and viral replication in vivo. For instance, a cohort of blood products recipients who acquired a Nef-mutant HIV-1 variant became long-term non-progressors [299]. Also, a SIV strain that was attenuated by mutating the nef gene proved to be a safe live attenuated vaccine in adult macaques, though a similar strain was pathogenic to neonatal macaques [300, 301]. In another study, nef transgenic mice developed AIDS-like presentation (immune suppression, wasting etc), suggesting that Nef could play a role in causing some of the AIDS-associated clinical manifestations [302].

Most of the effects of Nef have been attributed to either manipulation of the expression level of surface markers on or activation of immune cells.

Among the effects on surface marker expression, most notable is the downregulation of surface CD4 levels in infected $CD4^+$ T cells, a process that could enable efficient release of new viral particles [303]. Nef also downregulates MHC-I expression on infected T cells, leading to impaired killing of infected cells by cytotoxic T cells [304]. In fact, HLA-B, which has been shown to be more protective against HIV than HLA-A, has also been shown to be less susceptible to Nef-mediated downregulation, suggesting that the effect of Nef on HLA could be significant in vivo [305]. In addition to the above effects on $CD4^+$ T cells, Nef affects expression of MHC-II, CD80 and CD86 on infected antigen presenting cells (APCs), an effect that could compromise the activation of T cells by the APCs [306, 307]. It also upregulates the expression of DC-SIGN on infected dendritic cells and therefore promotes lymphocyte clustering and viral spread [308].

Additionally, HIV-1 Nef has been shown to modulate the activation of immune

cells. This has been mainly attributed to its ability to interact with the NFκB pathway in T cells and macrophages. Most important are the direct and indirect activation signals that it delivers to T cells hence increasing viral infectivity and viral replication. The protein has been shown to lower the threshold for TCR and CD28 mediated activation of T cells [21]. It also mediates the release of soluble inflammatory factors from macrophages that in turn act as chemokines and activation signals to T cells, drawing the T cells to sites of HIV replication and activating them to make them permissive to infection [309, 310]. Additional soluble factors such as soluble ICAM and soluble CD23 are released from Nef expressing macrophages and render B cells stimulatory to T cells [311].

In addition, HIV-1 Nef has been shown to reduce host T-cell actin dynamics following TCR engagement [312]. That finding concurs with the observed defective homing of Nef-expressing T cells to lymph nodes in a mouse model [313]. Other effects include cytotoxicity on uninfected T cells (effect of exogenous Nef) [314] and impairment of phagocytosis in macrophages [315].

HIV-1 Nef has also been shown to indirectly contribute to B-cell activation by inducing the release of ferritin from infected macrophages. The ferritin then interacts with B cells to cause polyclonal activation [133]. On the other hand, it has been shown to impair CD40L mediated class switching in vitro, implying that it could benefit the virus by blunting the antiviral antibody response. The ability to impair class switching while activating the NFκB pathway of immune cells is somehow contradictory. However, it was shown that the activation of NFκB pathway on Nef-exposed B cells was relatively inefficient, leading to engagement of negative feedback mechanisms that rendered the B cells refractory to subsequent CD40L stimulation [25]. Indeed, expression of Nef in drosophila inhibits relic NFκB dependent innate immune responses [316].

This chapter presents experiments that were done with the aim of determining the effect of various combinations of stimulants on B cells and the in vitro effect of exogenous HIV-1 Nef on B-cell responses to such stimulant-combinations.

6.2 Results

6.2.1 Titration of reagents

All stimulants were individually titrated to establish optimal stimulant concentrations for the experiment. Concentrations of stimulants were plotted against the magnitude of the various B cell responses that they elicited. The concentrations that were chosen were those that lay on the part of the curve where a unit increment in stimulant concentration corresponded with maximal increment in B-cell response i.e. the exponential part of the curve. This was done to enable the combination of different stimulants without eliciting saturating responses that would mask any effect of adding other stimulants in the combination or mask any immunomodulatory effects of recombinant HIV-1 Nef.

Titration of CpG-B (ODN-2006)

Oligodeoxynucleotide (ODN) 2006 was used in the reported experiments. It is a CpG-B/K-type ODN that has a full phosphorothioate backbone that protects it from degradation by nucleases. Its sequence is 5'-**tcgtcg**ttttgt**cg**ttttgt**cg**tt-3' whereby bold letters represent the CpG dinucleotides and the regular letters represent phosphorothioate linkage. ODN-2006 is a potent ligand for TLR9. The TLR9 generally

detects unmethylated cytosine-phosphate-guanosine (CpG) motifs that are common within bacterial DNA but relatively underrepresented in mammalian DNA. Like other compounds containing CpG motifs, ODN-2006 is highly effective in stimulating B cells from humans and mice among other animals and can therefore be used to assess the effect of bacterial DNA on B cells [317, 318].

In the titration experiments, the optimal concentration of the ODN-2006, simply referred to as CpG, as determined on the basis of day 8 IgG and IgM ELISA optical density values was 1μg/ml. The flow cytometry assays for determination of proliferation and differentiation of B cells into plasmablasts had not been optimised as at that time.

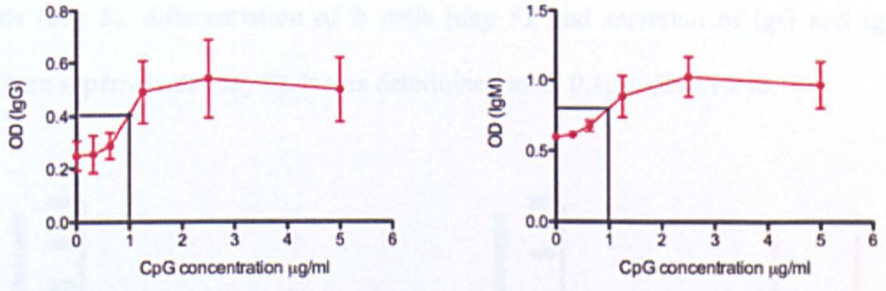

Figure 6.1: Titration of CpG. The most suitable concentration of CpG was established at 1μg/ml. Data represents two experiments. Symbols show mean ±SEM.

Titration of R848

R848 is a low molecular weight imidazoquinoline molecule that that has a molecular weight of 314 g/mole and a molecular formula $C_{17}H_{22}N_4O_2$. It was initially investigated for its potent antiviral and antitumor activity, and has since been shown to be a potent activator of immune cells via the TLR7 and TLR8 receptors whose natural ligand is

viral single stranded RNA [319]. R848 is particularly effective for activating B cells and can be used to mimic the effect of HIV-1 single stranded RNA [320].

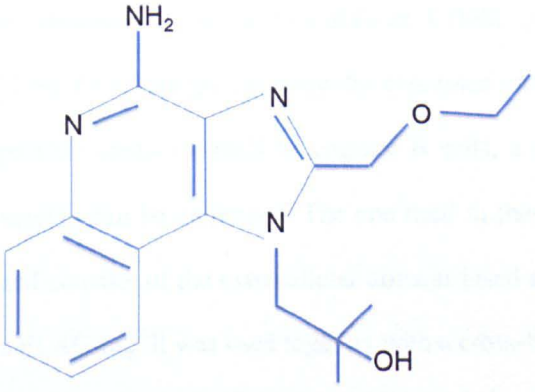

Figure 6.2: Chemical structure of R848

The optimal concentration of R848 was determined on the basis of proliferation of B cells (day 5), differentiation of B cells (day 5), and secretion of IgG and IgM into culture supernatants (day 8). It was determined to be 0.1μg/ml of R848.

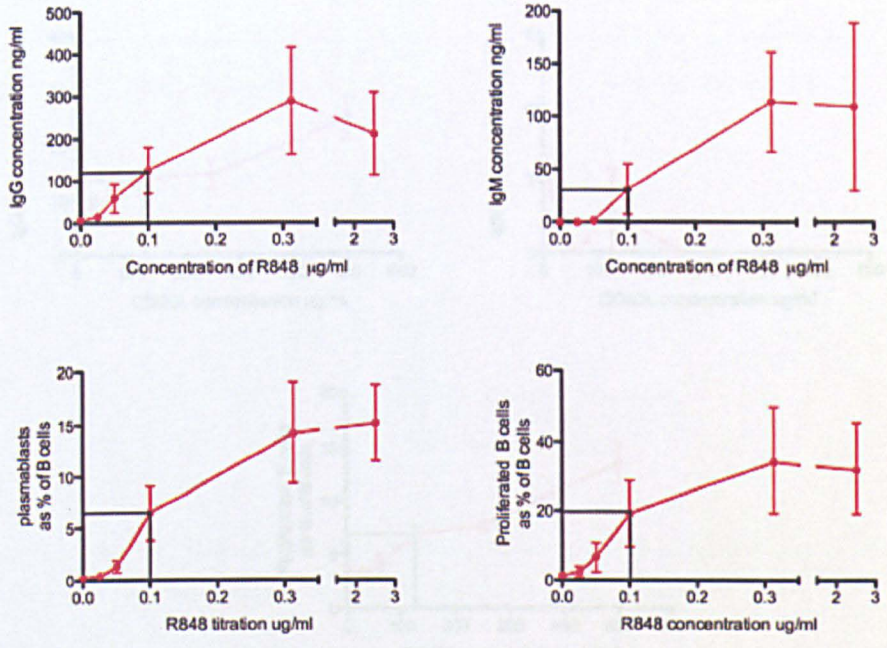

Figure 6.3: Titration of R848. The most suitable concentration of R848 was established at 0.1μg/ml.

Data represents two experiments. Symbols show mean ±SEM.

Titration of CD40L

As discussed earlier in this chapter, CD40L (CD154) is the natural ligand for CD40 [189]. Even though it is naturally expressed on the surface of activated helper T cells to provide contact signals to cognate B cells, a recombinant biologically active soluble version can be generated. The one used in these experiments was generated in *E. coli* and consists of the extracellular domain fused at the N-terminus to a linker peptide and a FLAG-tag. It was used together with a cross-linking enhancer.

The optimal concentration of the CD40L was determined on the basis of proliferation of B cells (day 5) and secretion of IgG and IgM into culture supernatants (day 8). It was determined to be 125 ng/ml. There was neither detectable differentiation into plasmablasts as measured by flow cytometry nor secretion of detectable IgM into culture supernatants when PBMCs were stimulated with CD40L alone.

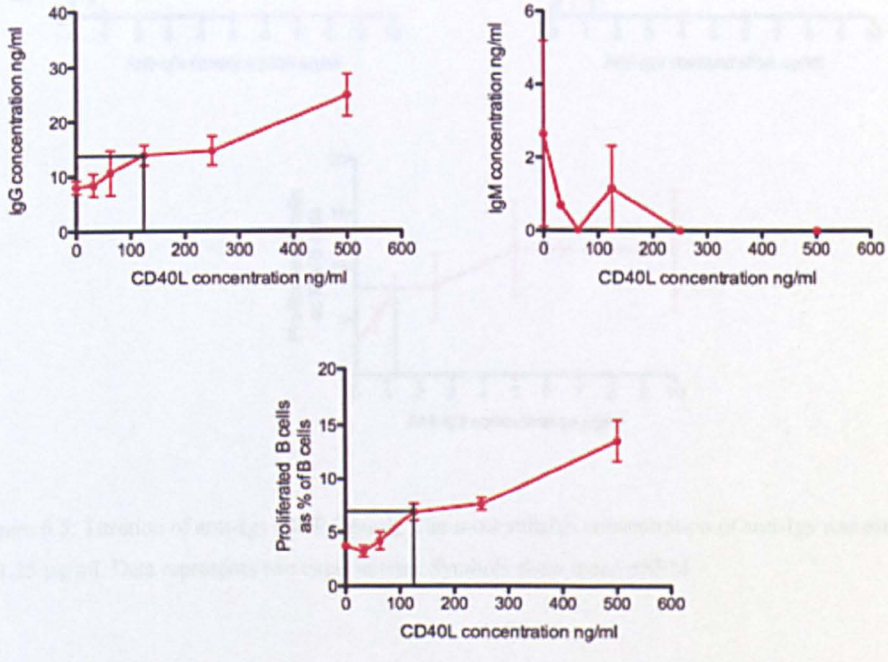

Figure 6.4: Titration of CD40L. The most suitable concentration of CD40L was established at 125

ng/ml. Data represents two experiments. Symbols show mean ±SEM.

Titration of anti-Igs (BCR ligand)

F(ab')2 fragment goat anti-human IgA+IgG+IgM were used to stimulate the B cells via the BCR. The optimal concentration of the anti-Igs (BCR ligand) was determined on the basis of proliferation of B cells (day 5) and secretion of IgM into culture supernatants (day 8). It was determined to be 1.25 μg/ml. There was neither detectable secretion of IgG into supernatants nor detectable differentiation into plasmablasts as measured by flow cytometry when PBMCs were stimulated with anti-Igs alone.

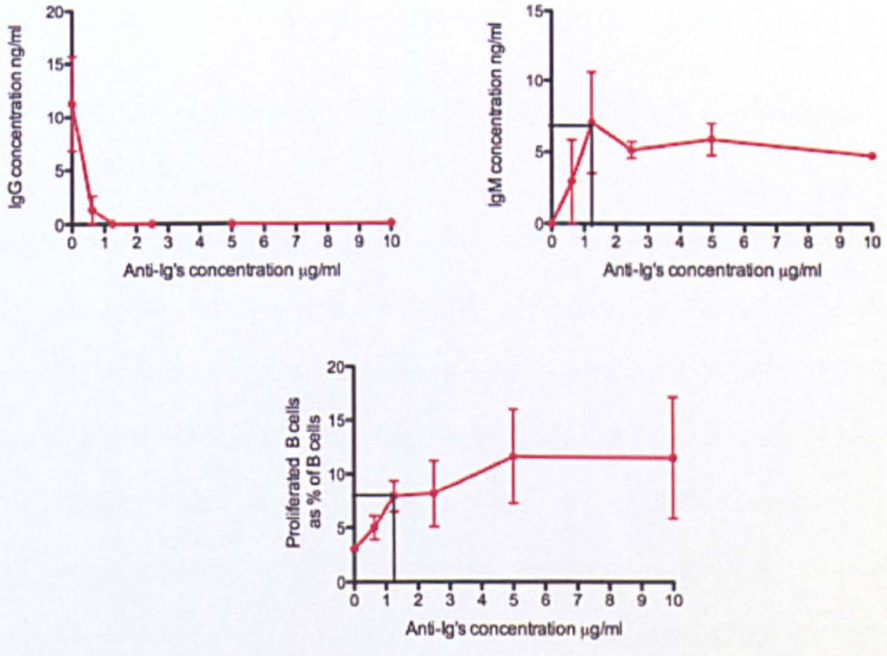

Figure 6.5: Titration of anti-Igs (BCR ligand). The most suitable concentration of anti-Igs was established at 1.25 μg/ml. Data represents two experiments. Symbols show mean ±SEM.

6.2.2 Combinations of different stimulants used

10 stimulant combinations were then made using the following combinations in order to mimic the different circumstances under which B cells may be stimulated in vivo during B-cell responses against complex pathogens and antigens. IL21 was used at 50ng/ml as per previous reports [210].

The effects of the various stimulant-combinations on B cells were first assessed by comparing samples that were pre-incubated with media alone. The effect of pre-incubation with recombinant HIV-1 proteins was then assessed by comparing samples that were pre-incubated with the wild-type HIV-1 Nef with samples that were pre-incubated with mutant HIV-1 Nef or media alone.

Stimulant-combination	IL21	CD40L	BCR-ligand (anti-Igs)	CpG	R848
1	√				
2	√		√		
3	√	√			
4	√	√	√		
5	√				√
6	√	√			√
7	√	√	√		√
8	√			√	
9	√	√		√	
10	√	√	√	√	

Table 6.1: Combinations of stimulants used to make the various stimulant-combinations.
√ indicates the use of the particular stimulant in the corresponding combination.

6.2.3 Proliferation of B cells after 5 days in response to the various stimulant combinations.

When the proliferation of B cells after exposure to the various stimulant combinations was evaluated by CFSE staining, differential effects were observed. Generally, the number of stimulants in culture correlated with the frequency of proliferating B cells, suggesting synergistic effect. The combination of CpG+IL21 elicited higher frequencies of proliferating B cells when compared with IL21 alone. Similarly, the combination of CD40L+IL21 elicited a trend of higher frequencies of proliferating B cells when compared with IL21 alone, though the difference was not statistically significant. R848+CD40L+BCR+IL21 elicited significantly higher frequencies of proliferating B cells than R848+CD40L+IL21 that in turn elicited significantly higher frequencies of proliferating B cells than R848+IL21, suggesting that addition of each stimulant synergised the effect of the other stimulants in the combination. The same effect was

observed in stimulants that contained CpG whereby CpG+CD40L+BCR+IL21 elicited significantly higher frequencies of proliferating B cells than CpG+CD40L+IL21 that in turn elicited significantly higher frequencies of proliferating B cells than CpG+IL21. The addition of TLR ligands enhanced proliferation in all cases. For instance, addition of R848 onto CD40L+IL21 or CD40L+BCRL+IL21 led to significantly higher proliferation. The same was observed upon adding CpG onto CD40L+IL21 or CD40L+BCRL+IL21. However, addition of BCRL onto CD40L+IL21 had no effect.

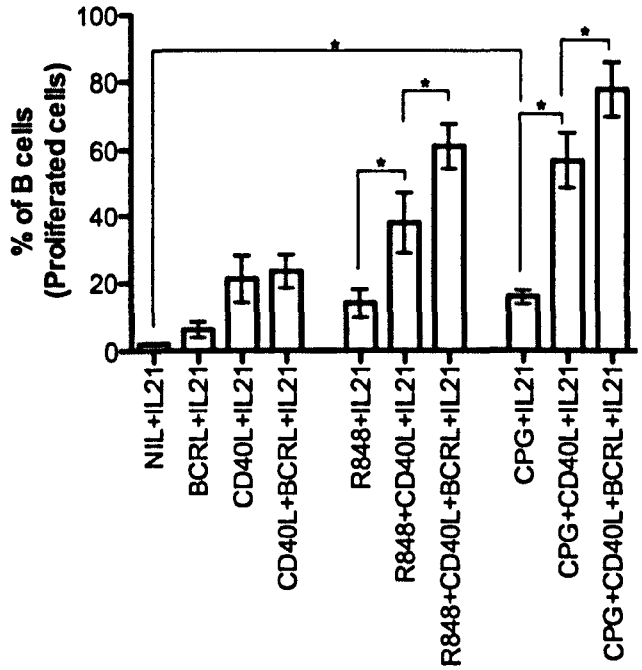

Figure 6.6: Frequencies of proliferating B cells after exposure to various stimulant-combinations. Data represents four independent experiments. Bars show mean ±SEM. * represents $P<0.05$.

6.2.4 Differentiation of B cells into plasmablasts after 5 days in response to the various stimulant-combinations.

When the differentiation of B cells into plasmablasts following stimulation with the various combinations was evaluated by flow cytometry, differential effects were also

observed. Generally, the number of stimulants in culture correlated with the frequency of plasmablasts, suggesting that the stimulants synergized with each other. For instance, CD40L+BCRL+IL21 elicited significantly higher frequencies of plasmablasts than BCRL+IL21. Similarly, R848+CD40L+BCRL+IL21 elicited significantly higher frequencies of plasmablasts than R848+CD40L+IL21, which in turn elicited higher frequencies of plasmablasts than R848+IL21. The same was observed with combinations that contained CpG whereby CpG+CD40L+IL21 elicited significantly higher frequencies of plasmablasts than CpG+IL21, which in turn elicited higher frequencies of plasmablasts than IL21 alone, suggesting that the stimulants synergised each other. The addition of TLR ligands enhanced differentiation into plasmablasts in all cases. For instance, addition of R848 onto CD40L+IL21 or CD40L+BCRL+IL21 led to significantly higher frequencies of plasmablasts. The same was observed upon adding CpG onto CD40L+IL21 or CD40L+BCRL+IL21. However, addition of BCRL onto CD40L+IL21 had no effect.

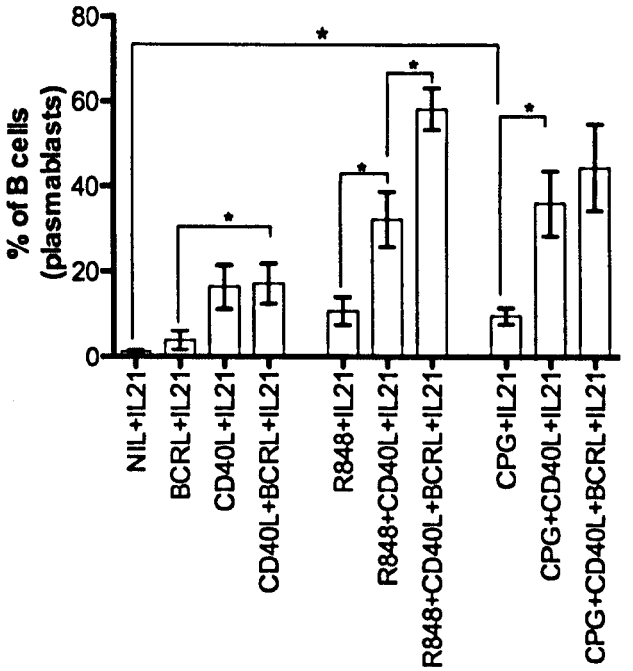

Figure 6.7: Frequencies of plasmablasts after exposure to various stimulant-combinations. Data represents four independent experiments. Bars show mean ±SEM. * represents $P<0.05$.

6.2.5 Expression of CD86 on B cells after 48 hours in response to the various stimulant-combinations.

The expression of CD86 on B cells as a marker of B-cell activation was evaluated by flow cytometry. There was differential expression of CD86 after stimulation with the various stimulants. The combination of CD40L+BCRL+IL21 elicited significantly higher frequencies of CD86+ B cells when compared with CD40L+IL21, which in turn elicited significantly higher frequencies of CD86+ B cells when compared with IL21 alone. Similarly, R848+CD40L+IL21 elicited significantly higher frequencies of CD86+ B cells when compared with R848+IL21. The same was observed with combinations containing CpG whereby CpG+CD40L+IL21 elicited significantly higher frequencies of CD86+ B cells when compared with CpG+IL21, which in turn

elicited significantly higher frequencies of CD86+ B cells when compared with IL21 alone, suggesting that the various stimulants synergised each other. Notably, R848+CD40L+BCRL+IL21 was comparable to R848+CD40L+IL21 while CpG+CD40L+BCRL+IL21 was comparable to CpG+CD40L+IL21, suggesting that addition of BCR ligand to combinations that had TLR ligands and CD40L did not have any additional effect on the expression of CD86 on B cells.

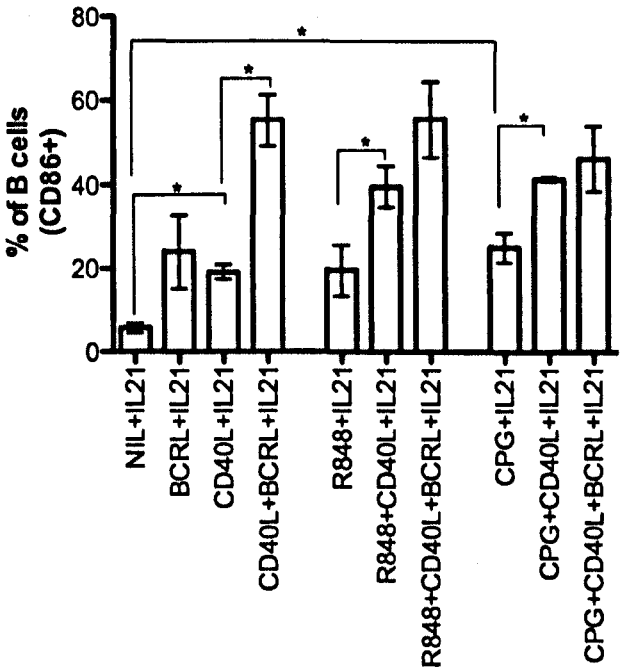

Figure 6.8: Frequencies of $CD86^{+}$ B cells after exposure to various stimulant-combinations. Data represents four independent experiments. Bars show mean ±SEM. * represents $P<0.05$.

6.2.6 Secretion of IgG into culture supernatants after 8 days in response to the various stimulant-combinations

When the secretion of IgG was evaluated by ELISA on culture supernatants, there was a notable high variability within each culture condition, probably because numbers of B cells vary between donors. This could have in turn resulted in generation of varied numbers of antibody secreting cells for samples from different donors. To eliminate

this bias, IgG indices, ratios of IgG elicited by various stimulants combinations to IgG elicited by a reference stimulant combination were calculated and also used to compare the performance of the various stimulants combinations. CD40L+IL21 was chosen as the reference stimulant because it is the best-studied model of B-cell responses and elicited responses that, unlike IL21 alone or BCRL+IL21, were not just background responses that could wrongly bias the proportions.

When the actual concentrations of IgG secreted into supernatants were compared between different stimulant-conditions, the only significant differences were between CpG+CD40L+IL21 and IL21 alone. Trends of differences between other conditions were observed but were not statistically significant, probably due to the high variability within stimulants. When the IgG indices were compared, differential secretion of IgG was observed among the various stimulant-conditions. Notably, addition of BCR ligand to any stimulant-combination dampened the secretion of IgG. For instance, BCRL+IL21 elicited significantly lower IgG indices when compared with IL21 alone, CD40L+BCRL+IL21 elicited significantly lower IgG indices when compared with CD40L+IL21 and R848+CD40L+BCRL+IL21 elicited significantly lower IgG indices when compared with R848+CD40L+IL21. TLR ligands and CD40L synergised with IL21 to induce secretion of IgG as shown by significantly higher IgG indices in CD40L+IL21, R848+IL21 and CpG+IL21 when they were compared with IL21 alone.

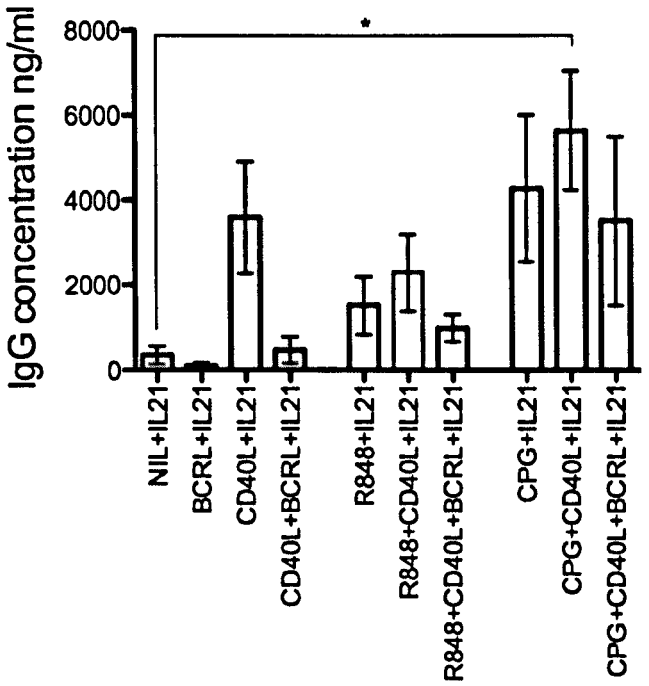

Figure 6.9: Concentrations of IgG secreted into culture supernatants after exposure of PBMCs to various stimulant-combinations. Data represents four independent experiments. Bars show mean ±SEM. * represents $P<0.05$.

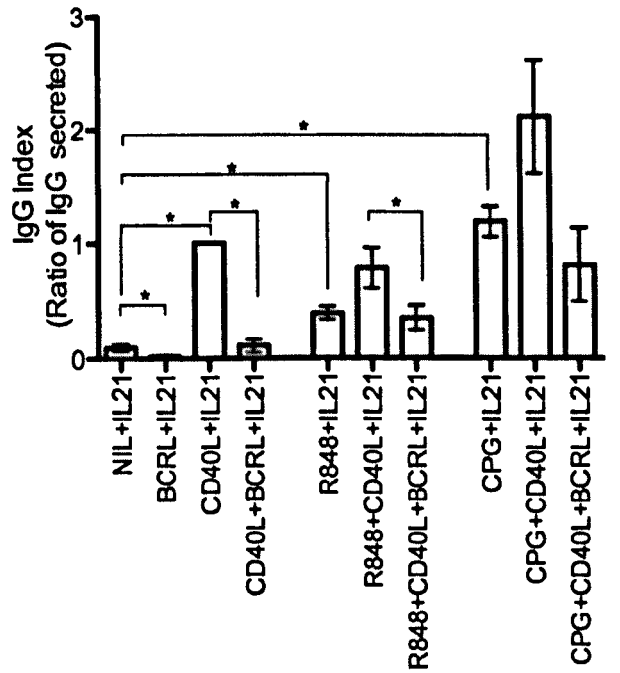

Figure 6.10: Indices of concentrations of IgG secreted into culture supernatants after exposure of

PBMCs to various stimulant-combinations. Data represents four independent experiments. Bars show mean ±SEM. * represents $P<0.05$.

6.2.7 Secretion of IgM into culture supernatants after 8 days in response to the various stimulants combinations

There was high variability within culture conditions in the amounts of IgM produced, probably because of variability of B-cell frequencies in the different donors. To minimise this bias, IgM index was calculated as a ratio of concentration of IgM in culture supernatants of various stimulant-combinations to concentration of IgM in culture supernatants of a reference condition (CpG+IL21). CpG+IL21 was chosen here instead of CD40L+IL21 as the reference condition because there was no secretion of IgM after stimulation with CD40L+IL21. The IgM index was then used to compare the performance of different stimulant-combinations.

There was little or undetectable IgM secretion in the stimulant-conditions that did not contain CpG. In those conditions that contained CpG, there were no significant differences in IgM secretion between various stimulants combinations. The same was observed when IgM indices were compared; there were no differences in IgM indices between various stimulants combinations except in the case of CpG+IL21 versus IL21 alone where the significant difference might have been driven by the lack of variability within the reference condition CpG+IL21(all donors had an IgM index of 1 for CpG+IL21).

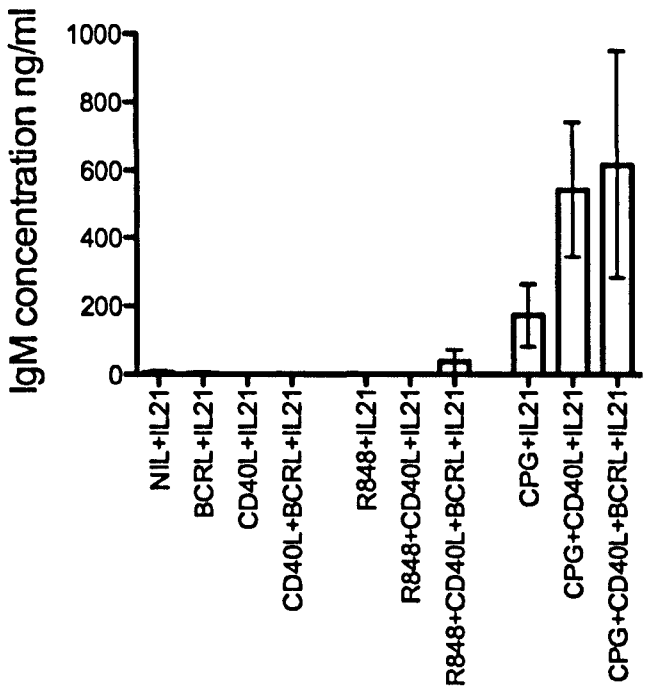

Figure 6.11: Concentrations of IgM secreted into culture supernatants after exposure of PBMCs to various stimulant-combinations. Data represents four independent experiments. Bars show mean ±SEM. * represents $P<0.05$.

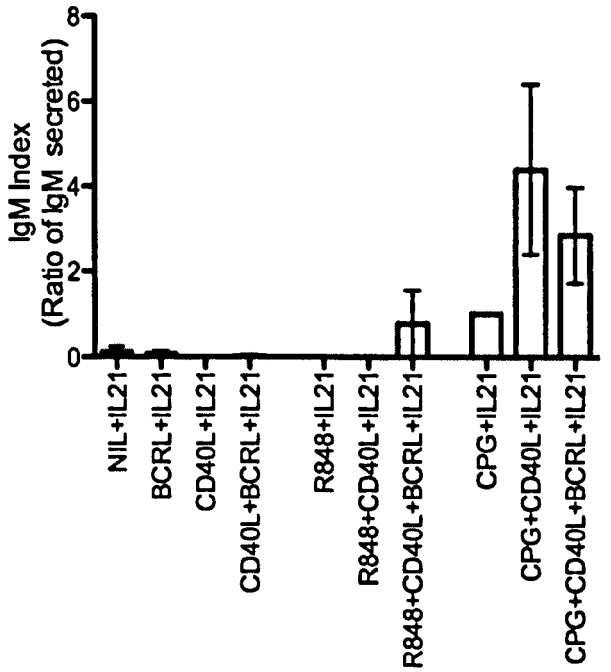

Figure 6.12: Indices of concentrations of IgM secreted into culture supernatants after exposure of PBMCs to various stimulant-combinations. Data represents four independent experiments. Bars show mean ±SEM. * represents $P<0.05$.

6.2.8 Secretion of TNF-alpha into culture supernatants after 48 hours in response to the various stimulant-combinations

TLR ligands and CD40L have been shown to induce secretion of TNF-alpha by B cells [321, 322]. Furthermore, the secreted TNF-alpha can synergize with B-cell stimulants in inducing further B-cell responses [323]. The secretion of the cytokine upon stimulation with various stimulant-combinations was therefore assessed in this study. However, the same stimulants have been shown to induce secretion of TNF-alpha by monocytes [324], making it likely that the TNF-alpha detected in the culture supernatants came from B cells as well as other cell-types that constitute PBMCs.

A TNF-alpha index was calculated for each donor and each stimulant combination as a ratio between the concentration of TNF-alpha in culture supernatants of the various stimulant-combinations and the concentration of TNF-alpha in culture supernatants of a reference stimulant condition (CD40L+IL21). When actual TNF-alpha concentrations were considered, there was a significant difference between R848+CD40L+IL21 and R848+IL21 with regard to secretion of the cytokine into culture supernatants. However, no other differences were observed. When TNF-alpha indices were considered, the secretion of TNF-alpha was highly dependent on presence of CD40L in the stimulant preparation; stimulant combinations that contained CD40L elicited significantly higher TNF-alpha indices when compared with those that did not have CD40L. Furthermore, there were no statistically significant differences between culture conditions that contained CD40L with regard to TNF-alpha indices.

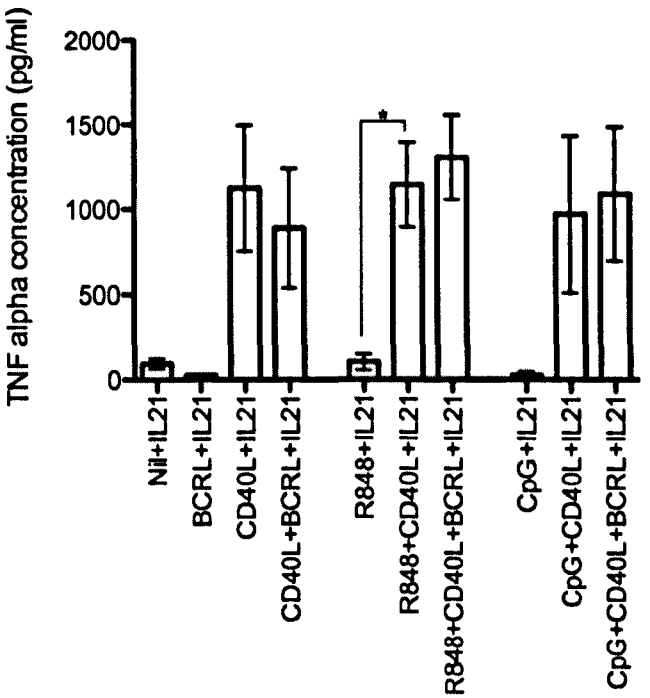

Figure 6.13: Concentrations of TNF-alpha secreted into culture supernatants after exposure of PBMCs to various stimulant-combinations. Data represents four independent experiments. Bars show mean ±SEM. * represents P<0.05.

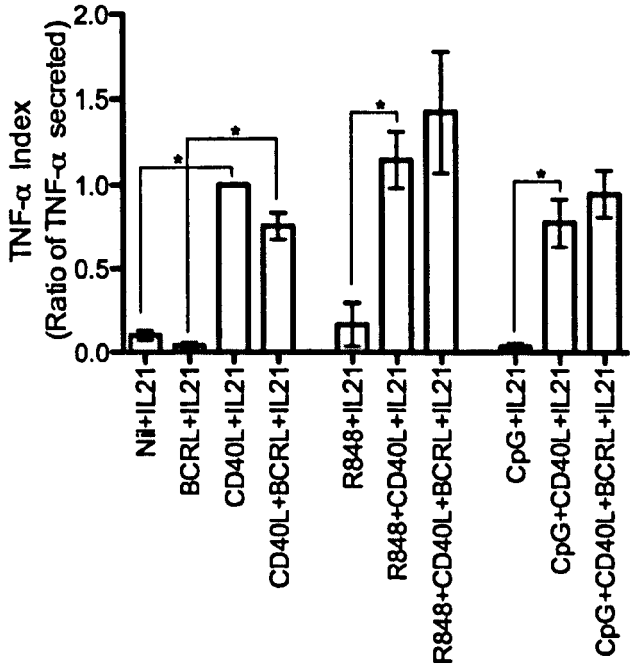

Figure 6.14: Indices of concentrations of TNF-alpha secreted into culture supernatants after exposure of PBMCs to various stimulant-combinations. Data represents four independent experiments. Bars show mean ±SEM. * represents $P<0.05$.

6.2.9 Secretion of IL6 into culture supernatants after 48 hours in response to the various stimulant-combinations

TLR ligands and CD40L have also been shown to induce secretion of IL6 by B cells [321, 322]. IL6 has been shown to potentiate the effect of B-cell stimulants in inducing B-cell responses [325]. The secretion of the cytokine upon stimulation with various stimulant-combinations was therefore also assessed in this study. However, stimulation of monocytes has been shown to make them secrete IL6 [326, 327], making it likely that the IL6 detected in the culture supernatants came from B cells as well as other

cell-types that constitute PBMCs.

There was high variability in IL6 concentrations in culture supernatants within the various stimulant-combinations, probably due to high variability in IL6 producing cells between donors. When the levels of IL6 were considered, there were no statistically significant differences in IL6 levels between various culture conditions.

An IL6 index was calculated for each donor and each stimulant-combination as a ratio between the concentration of IL6 in culture supernatants of the various stimulant-combinations and the concentration of IL6 in culture supernatants of a reference stimulant condition (CD40L+IL21). Notably, all culture conditions that contained CD40L had significantly higher IL6 indices when compared with corresponding culture conditions that did not have CD40L, suggesting that CD40L played an important role in determining the amount of IL6 produced by the cultured PBMCs. Presence of BCR ligand did not appear to affect the amount of IL6 produced.

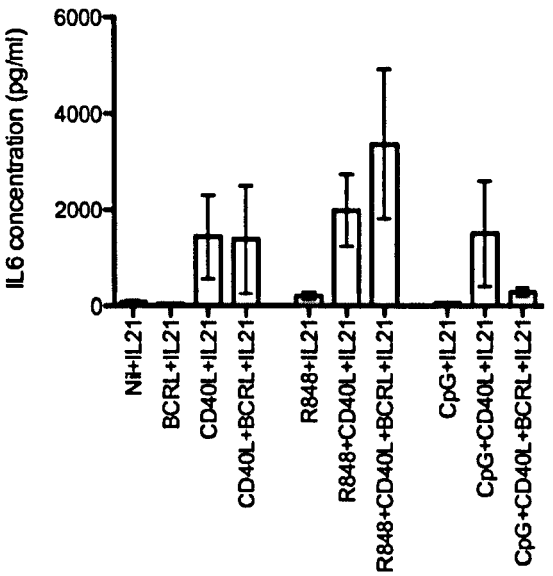

Figure 6.15: Concentrations of IL6 secreted into culture supernatants after exposure of PBMCs to various stimulant-combinations. Data represents four independent experiments. Bars show mean ±SEM. * represents P<0.05.

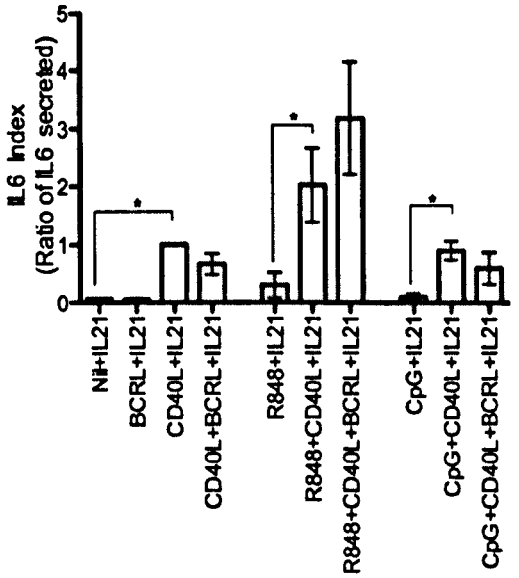

Figure 6.16: Indices of concentrations of IL6 secreted into culture supernatants after exposure of PBMCs to various stimulant-combinations. Data represents four independent experiments. Bars show mean ±SEM. * represents P<0.05.

6.2.10 Effect of recombinant HIV-1 Nef on proliferation of B cells after stimulation with various stimulants for 5 days

The effect of recombinant HIV-1 Nef on B-cell proliferation was assessed by comparing the proliferative response after pre-incubation with wild-type recombinant HIV-1 Nef, mutant non-myristoylated recombinant HIV-1 Nef and media alone. Notably, across all stimulant-combinations, there was no difference between the samples that were pre-incubated with recombinant HIV-1 Nef and those pre-incubated with mutant (non-myristoylated) HIV-1 Nef or media alone.

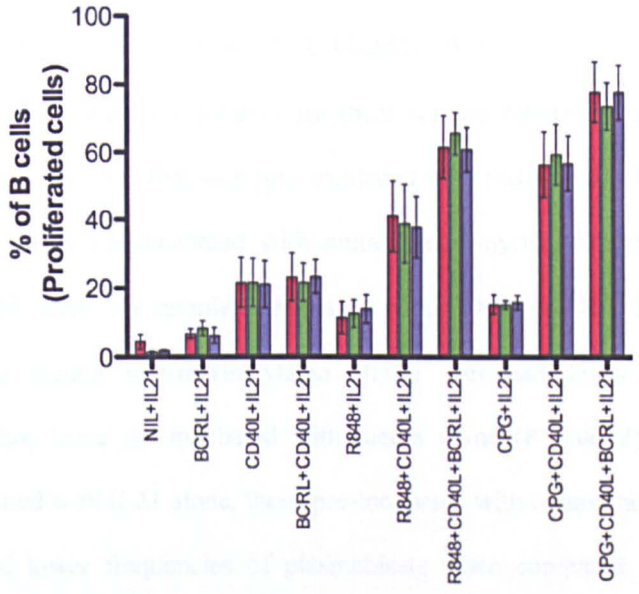

Figure 6.17: Frequencies of proliferating B cells after pre-incubation with HIV proteins and subsequent exposure to various stimulant-combinations. All samples were pre-incubated with recombinant HIV-1 Nef (red), recombinant mutant (non-myristoylated) HIV-1 Nef (Green) or media (Blue). Data represents four independent experiments. Bars show mean ±SEM.

Stimulant	Mean(standard error) of proliferating B cells			P value		
	Nef	MNef	Nil	Nef vs MNef	Nef vs Nil	MNef vs Nil
NIL+IL21	4.4(2.2)	1.3(0.2)	1.9(0.3)	0.2786	0.3001	0.1965
BCRL+IL21	6.8(1.5)	8.4(2.1)	6.3(2.3)	0.1993	0.6042	0.1430
CD40L+IL21	21.55(7.4)	21.66(7.0)	21.00(6.9)	0.8051	0.8893	0.8512
CD40L+BCRL+IL21	23.2(7.1)	21.7(5.6)	23.4(4.9)	0.5402	0.9510	0.3743
R848+IL21	11.4(4.7)	12.6(4.0)	13.9(4.0)	0.3611	0.0515	0.1403
R848+CD40L+IL21	41.2(10.4)	39.0(11.6)	37.9(9.1)	0.2718	0.1753	0.7148
R848+CD40L+BCR+IL21	61.7(9.4)	65.8(6.3)	61.1(6.6)	0.4569	0.8722	0.1881
CpG+IL21	15.1(2.8)	15.1(1.2)	15.8(2.1)	0.9886	0.6728	0.7902
CpG +CD40L+IL21	56.5(9.7)	59.4(9.0)	56.8(8.2)	0.3976	0.9076	0.4123
CpG+CD40L+BCRL+IL21	77.8(8.7)	73.7(7.0)	77.8(7.8)	0.4195	0.9786	0.3591

Table 6.2: Mean (and standard error of mean) of frequencies of proliferating B cells for the various stimulant cocktails after pre-incubation with wild-type HIV-1 Nef (Nef), mutant non-myristoylated HIV-1 Nef (MNef) or media (Nil) and the corresponding P values after pair-wise comparisons. Paired Student's T test was used.

6.2.11 Effect of recombinant HIV-1 Nef on differentiation of B cells into plasmablasts after stimulation with various stimulants for 5 days

Notably, across all stimulant-combinations, there was no difference in frequencies of plasmablasts when samples that were pre-incubated with recombinant HIV-1 Nef were compared with those pre-incubated with mutant (non-myristoylated) HIV-1 Nef or media alone. However, for samples stimulated with CD40L+BCRL+IL21, those pre-incubated with mutant non-myristoylated HIV-1 Nef had lower frequencies of plasmablasts than those pre-incubated with media alone (P=0.0052). Similarly, for samples stimulated with IL21 alone, those pre-incubated with mutant non-myristoylated HIV-1 Nef had lower frequencies of plasmablasts when compared with those pre-incubated with media (P=0.0357).

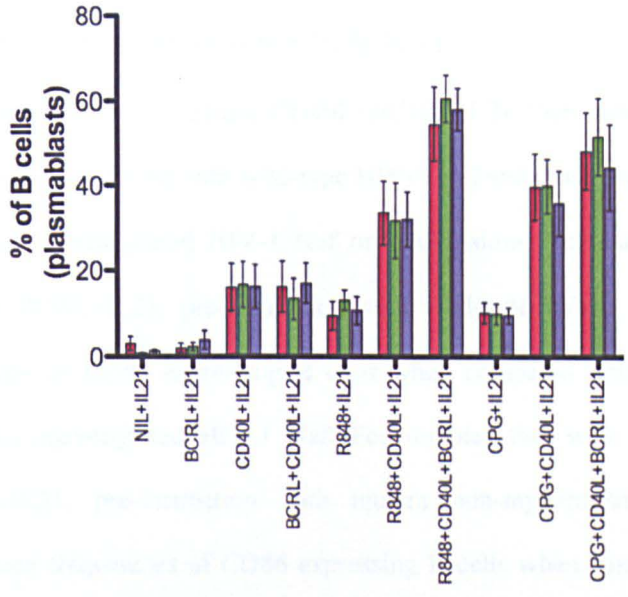

Figure 6.18: Frequencies of plasmablasts after pre-incubation with HIV proteins and subsequent exposure to various stimulant-combinations. All samples were pre-incubated with recombinant HIV-1 Nef (red), recombinant mutant (non-myristoylated) HIV-1 Nef (Green) or media (Blue). Data represents four independent experiments. Bars show mean ±SEM.

Stimulant	Mean(standard error) % of plasmablasts			P value		
	Nef	MNef	Nil	Nef vs MNef	Nef vs Nil	MNef vs Nil
NIL+IL21	3.05(1.7)	0.70(0.2)	1.29(0.3)	0.2700	0.3587	0.0357
BCRL+IL21	1.95(1.1)	2.27(0.9)	3.94(2.3)	0.3545	0.5242	0.5954
CD40L+IL21	16.07(5.5)	16.70(5.3)	16.29(5.1)	0.5591	0.9371	0.8138
CD40L+BCRL+IL21	16.20(5.9)	13.43(4.8)	17.07(4.7)	0.3280	0.7136	0.0052
R848+IL21	9.50(3.3)	13.05(2.3)	10.71(3.1)	0.2342	0.2734	0.3236
R848+CD40L+IL21	33.60(7.5)	31.75(9.0)	32.13(6.5)	0.3806	0.2709	0.9035
R848+CD40L+BCR+IL21	54.73(8.7)	60.73(5.4)	58.20(4.9)	0.2528	0.4708	0.2083
CpG+IL21	10.08(2.4)	9.88(2.4)	9.47(1.8)	0.9485	0.5468	0.8493
CpG +CD40L+IL21	39.73(7.9)	39.88(6.5)	35.88(7.7)	0.9572	0.1491	0.0552
CpG+CD40L+BCRL+IL21	48.25(9.2)	51.63(9.1)	44.38(10.3)	0.5346	0.4734	0.2633

Table 6.3: Mean (and standard error of mean) of frequencies of plasmablasts for the various stimulant-combinations after pre-incubation with wild-type HIV-1 Nef (Nef), mutant non-myristoylated HIV-1 Nef (MNef) or media (Nil) and the corresponding P values after pair-wise comparisons. Paired Student's T test was used. Significant P values are shown in red text.

6.2.12 Effect of recombinant HIV-1 Nef on expression of CD86 on B cells after stimulation with various stimulants for 48 hours

With the exception of BCRL+IL21 and CD40L+BCRL+IL21, there were no differences between samples pre-incubated with wild-type HIV-1 Nef and those pre-incubated with either mutant non-myristoylated HIV-1 Nef or media alone. For samples that were stimulated with BCRL+IL21, pre-incubation with wild-type HIV-1 Nef resulted in higher frequencies of CD86 expressing B cells when compared with pre-incubation with mutant non-myristoylated HIV-1 Nef. For samples that were stimulated with CD40L+BCRL+IL21, pre-incubation with mutant non-myristoylated HIV-1 Nef resulted into lower frequencies of CD86 expressing B cells when compared with pre-incubation with either wild-type HIV-1 Nef or media alone.

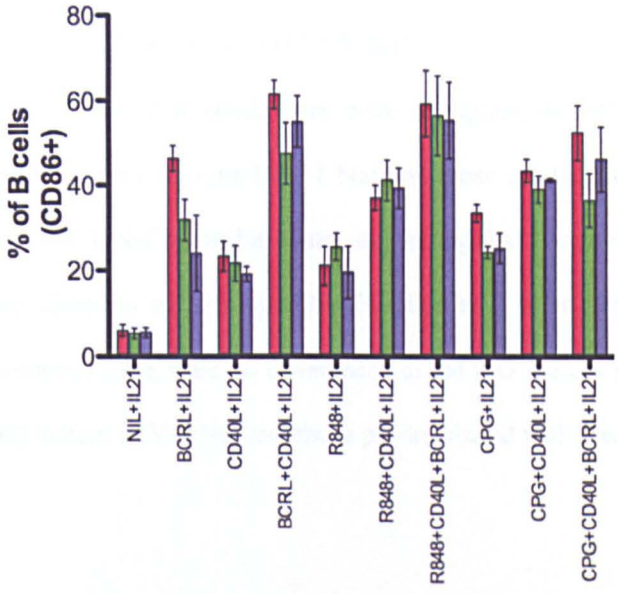

Figure 6.19: Frequencies of CD86 expressing B cells after pre-incubation with HIV proteins and subsequent exposure to various stimulant-combinations. All samples were pre-incubated with recombinant HIV-1 Nef (red), recombinant mutant (non-myristoylated) HIV-1 Nef (Green) or media (Blue). Data represents four independent experiments. Bars show mean ±SEM.

Stimulant	Mean(standard error) % of CD86+ B cells			P value		
	Nef	MNef	Nil	Nef vs MNef	Nef vs Nil	MNef vs Nil
NIL+IL21	6.12(1.4)	5.42(1.2)	5.65(1.1)	0.4148	0.6327	0.5337
BCRL+IL21	46.45(3.0)	31.80(4.9)	23.97(8.9)	0.0089	0.0725	0.4554
CD40L+IL21	23.18(3.5)	21.58(4.1)	19.10(1.8)	0.2045	0.1306	0.4032
CD40L+BCRL+IL21	61.63(3.4)	47.68(7.4)	55.23(6.0)	0.0483	0.1716	0.0308
R848+IL21	21.09(4.6)	25.53(3.2)	19.54(6.1)	0.5449	0.8514	0.3026
R848+CD40L+IL21	37.10(3.0)	41.35(4.8)	39.48(4.8)	0.2762	0.7182	0.7650
R848+CD40L+BCR+IL21	59.40(7.8)	56.63(9.4)	55.58(9.0)	0.8003	0.4027	0.9145
CpG+IL21	33.43(2.1)	24.18(1.5)	25.18(3.6)	0.0711	0.2423	0.7398
CpG +CD40L+IL21	43.50(2.8)	39.08(3.2)	41.25(0.4)	0.3369	0.4121	0.5569
CpG+CD40L+BCRL+IL21	52.48(6.5)	36.53(6.4)	46.28(7.6)	0.1671	0.1295	0.3654

Table 6.4: Mean (and standard error of mean) of frequencies of CD86 expressing B cells for the various stimulant-combinations after pre-incubation with wild-type HIV-1 Nef (Nef), mutant non-myristoylated HIV-1 Nef (MNef) or media (Nil) and the corresponding P values after pair-wise comparisons. Paired Student's T test was used. Significant P values are shown in red text.

6.2.13 Effect of recombinant HIV-1 Nef on secretion of IgG by PBMCs after stimulation with various stimulants for 8 days

When the IgG indices were compared, there were no significant differences between samples pre-incubated with wild-type HIV-1 Nef and those pre-incubated with mutant non-myristoylated HIV-1 Nef or media alone, suggesting that recombinant HIV-1 Nef did not affect the secretion of IgG by PBMCs stimulated with different stimulants combinations. Similarly, there were no differences in the IgG indices between samples pre-incubated with mutant HIV-1 Nef and those pre-incubated with media alone.

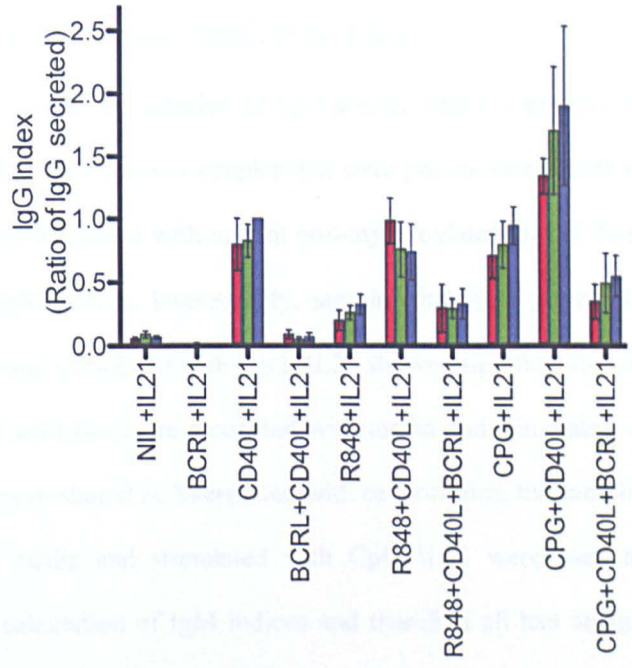

Figure 6.20: IgG Indices after pre-incubation with HIV proteins and subsequent exposure of PBMCs to various stimulant-combinations. All samples were pre-incubated with recombinant HIV-1 Nef (red), recombinant mutant (non-myristoylated) HIV-1 Nef (Green) or media (Blue). Data represents four independent experiments. Bars show mean ±SEM.

Stimulant	Mean (standard error) IgG Index			P value		
	Nef	MNef	Nil	Nef vs MNef	Nef vs Nil	MNef vs Nil
NIL+IL21	0.06(0.01)	0.09(0.03)	0.07(0.01)	0.1491	0.4003	0.2805
BCRL+IL21	0.00(0.00)	0.01(0.01)	0.01(0.01)	0.2890	0.1985	0.6767
CD40L+IL21	0.80(0.21)	0.82(0.13)	1.00(0.00)	0.9241	0.3974	0.2704
CD40L+BCRL+IL21	0.09(0.04)	0.05(0.02)	0.07(0.03)	0.3201	0.6497	0.3448
R848+IL21	0.20(0.08)	0.26(0.05)	0.32(0.08)	0.2320	0.0833	0.3855
R848+CD40L+IL21	0.99(0.18)	0.76(0.22)	0.74(0.22)	0.1407	0.1495	0.6038
R848+CD40L+BCR+IL21	0.30(0.19)	0.29(0.08)	0.33(0.12)	0.9838	0.9003	0.6884
CpG+IL21	0.70(0.17)	0.80(0.19)	0.95(0.15)	0.2658	0.1958	0.2292
CpG +CD40L+IL21	1.34(0.14)	1.70(0.51)	1.90(0.64)	0.4536	0.3805	0.2479
CpG+CD40L+BCRL+IL21	0.35(0.14)	0.49(0.23)	0.54(0.16)	0.4897	0.2894	0.6547

Table 6.5: Mean (and standard error of mean) of IgG indices for the various stimulant-combinations after pre-incubation with wild-type HIV-1 Nef (Nef), mutant non-myristoylated HIV-1 Nef (MNef) or media (Nil) and the corresponding P values after pair-wise comparisons. Paired Student's T test was used.

6.2.14 Effect of recombinant HIV-1 Nef on secretion of IgM by PBMCs after stimulation with various stimulants for 8 days

Similar to observations in secretion of IgG above, with the exception of CpG+IL21, there was no difference between samples that were pre-incubated with wild-type HIV-1 Nef and those pre-incubated with mutant non-myristoylated HIV-1 Nef or media alone with regard to IgM indices. Interestingly, samples that were pre-incubated with wild-type HIV-1 Nef and stimulated with CpG+IL21 showed significantly lower IgM indices when compared with those pre-incubated with media and stimulated with CpG+IL21. However, this result should be interpreted with caution since the samples that were pre-incubated with media and stimulated with CpG+IL21 were used as the reference samples in the calculation of IgM indices and therefore all had an IgM index of 1.0, with a standard error of 0.00 that could have driven the statistical significance.

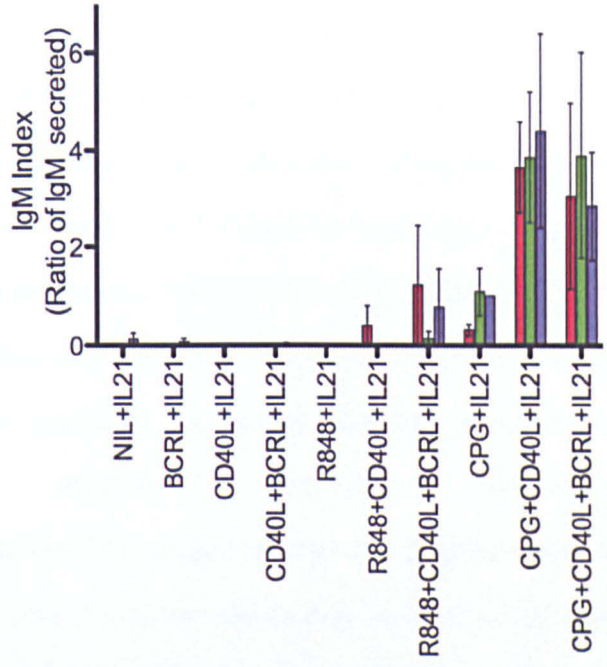

Figure 6.21: IgM indices after pre-incubation with HIV proteins and subsequent exposure to various stimulant-combinations. All samples were pre-incubated with recombinant HIV-1 Nef (red), recombinant mutant (non-myristoylated) HIV-1 Nef (Green) or media (Blue). Data represents four independent experiments. Bars show mean ±SEM.

Stimulant-combination	Mean(standard error) IgM Index			P value		
	Nef	MNef	Nil	Nef vs MNef	Nef vs Nil	MNef vs Nil
NIL+IL21	0	0	0.13(0.13)	0.3910	0.3910	
BCRL+IL21	0	0	0.07(0.07)	-	0.3910	0.3910
CD40L+IL21	0	0	0	-	-	-
CD40L+BCRL+IL21	0	0	0.03(0.03)	-	0.3910	0.3910
R848+IL21	0	0	0.00(0.00)	-	0.3910	0.3910
R848+CD40L+IL21	0.41(0.41)	0	0.01(0.01)	0.3910	0.4004	0.3910
R848+CD40L+BCR+IL21	1.22(1.22)	0.14(0.14)	0.78(0.78)	0.3910	0.8046	0.5021
CpG+IL21	0.31(0.12)	1.09(0.48)	1.00(0.00)	0.2675	0.0106	0.8691
CpG +CD40L+IL21	3.66(0.94)	3.86(1.36)	4.40(2.00)	0.8954	0.7278	0.4969
CpG+CD40L+BCRL+IL21	3.06(1.92)	3.90(2.12)	2.85(1.13)	0.7783	0.8613	0.6226

Table 6.6: Mean (and standard error of mean) of IgM indices for the various stimulant-combinations after pre-incubation with wild-type HIV-1 Nef (Nef), mutant non-myristoylated HIV-1 Nef (MNef) or media (Nil) and the corresponding P values after pair-wise comparisons. Paired Student's T test was used. Significant P values are shown in red text.

6.2.15 Effect of recombinant HIV-1 Nef on secretion of TNF-alpha by PBMCs after stimulation with various stimulants for 48 hours

Pre-incubation with wild-type recombinant HIV-1 Nef induced high and comparable TNF-alpha indices regardless of the stimulant combination, suggesting that wild-type HIV-1 Nef, independently from the stimulants used, causes secretion of TNF-alpha by PBMCs. Pre-incubation with wild-type HIV-1 Nef elicited higher TNF-alpha indices than pre-incubation with mutant non-myristoylated HIV-1 Nef but that difference was only statistically significant for samples stimulated with Nil+IL21, BCRL+IL21, CD40L+IL21, R848+IL21, CpG+IL21, CpG+CD40L+IL21 and CpG+CD40L+BCRL+IL21, suggesting that myristoylation was necessary for the observed effect. Likewise, pre-incubation with media alone led to significantly higher TNF-alpha indices than pre-incubation with mutant non-myristoylated HIV-1 Nef with regard to all stimulant-conditions except Nil+IL21, BCRL+IL21, R848+IL21 and CpG+IL21. Notably, these are the stimulant combinations that did not contain CD40L. The results suggest that non-myristoylated HIV-1 Nef could be having some inhibitory effects on CD40L-mediated activation of PBMCs to secrete the cytokine. Notably, the TNF-alpha indices after pre-incubation with media were higher than those induced after pre-incubation with wild-type HIV-1 Nef under all CD40L-containing stimulant-conditions although the difference was statistically significant only after simulation with R848+CD40L+BCR+IL21. This, coupled with the fact that pre-incubation with wild-type HIV-1 Nef induced higher TNF-alpha indices when compared to pre-incubation with media in conditions that did not contain CD40L, suggests that wild-type HIV-1 Nef protein induces some immune activation while at the same time inhibiting CD40L-mediated activation.

The possibility of contamination of the wild-type HIV-1 Nef protein with lipopolysaccharide (LPS) provides a possible alternative explanation for the

observed effects on TNF-alpha secretion. LPS has been shown to induce secretion of proinflammatory cytokines (like TNF-alpha and IL6) by monocytes [327]. Even though a previous publication reported that the Nef proteins were free of endotoxin [25], presence of trace amounts of LPS in the particular vials that were used cannot be ruled out.

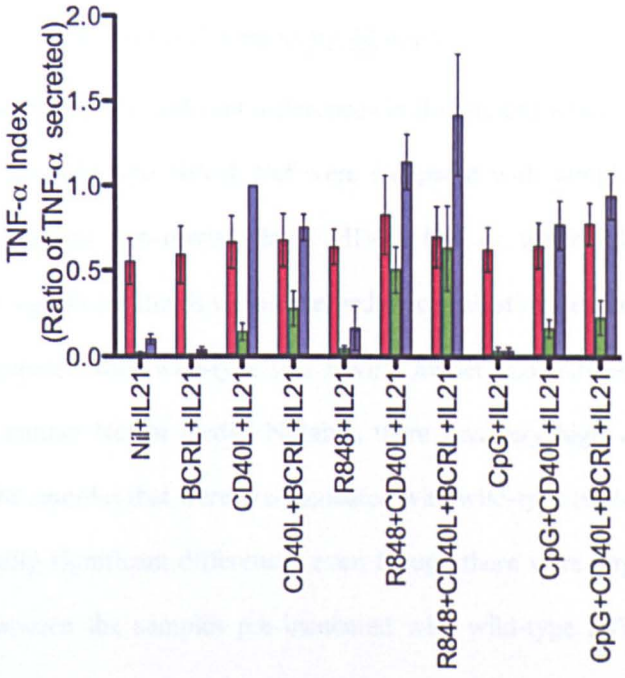

Figure 6.22: TNF-alpha indices after pre-incubation with HIV proteins and subsequent exposure to various stimulant-combinations. All samples were pre-incubated with recombinant HIV-1 Nef (red), recombinant mutant (non-myristoylated) HIV-1 Nef (Green) or media (Blue). Data represents four independent experiments. Bars show mean ±SEM.

Stimulant	Mean(standard error) TNF-α Index			P value		
	Nef	MNef	Nil	Nef vs MNef	Nef vs Nil	MNef vs Nil
NIL+IL21	0.55(0.13)	0.02(0.01)	0.10(0.03)	0.0281	0.0504	0.1038
BCRL+IL21	0.59(0.17)	0.01(0.01)	0.04(0.02)	0.0417	0.0344	0.2418
CD40L+IL21	0.67(0.16)	0.15(0.05)	1.00(0.00)	0.0277	0.1208	0.0004
CD40L+BCRL+IL21	0.68(0.16)	0.28(0.10)	0.75(0.08)	0.0874	0.6684	0.0452
R848+IL21	0.64(0.11)	0.05(0.02)	0.17(0.13)	0.0090	0.0033	0.36 98
R848+CD40L+IL21	0.83(0.24)	0.50(0.13)	1.15(0.16)	0.2222	0.2616	0.0173
R848+CD40L+BCR+IL21	0.70(0.18)	0.63(0.25)	1.42(0.36)	0.5900	0.0251	0.0162
CpG+IL21	0.62(0.13)	0.03(0.02)	0.03(0.02)	0.0124	0.0139	0.8882
CpG +CD40L+IL21	0.65(0.14)	0.17(0.05)	0.77(0.14)	0.0442	0.5974	0.0362
CpG+CD40L+BCRL+IL21	0.78(0.13)	0.23(0.10)	0.94(0.14)	0.0067	0.4936	0.0141

Table 6.7: Mean (and standard error of mean) of quantities of TNF-alpha produced for the various stimulant conditions after pre-incubation with wild-type HIV-1 Nef (Nef), mutant non-myristoylated HIV-1 Nef (MNef) or media (Nil) and the corresponding P values after pair-wise comparisons. Paired Student's T test was used. Significant P values are shown in red text.

6.2.16 Effect of recombinant HIV-1 Nef on secretion of IL6 by PBMCs after stimulation with various stimulants for 48 hours

There were no statistically significant differences in IL6 indices when samples that were pre-incubated with wild-type HIV-1 Nef were compared with samples that were pre-incubated with mutant non-myristoylated HIV-1 Nef or media alone. However, statistically non-significant trends were observed across all stimulant combinations with samples pre-incubated with wild-type Nef having higher IL6 indices than those pre-incubated with mutant Nef or media. Notably, there was very high variability in IL6 indices within the samples that were pre-incubated with wild-type HIV-1 Nef, hence the lack of statistically significant differences even though there were large differences in mean values between the samples pre-incubated with wild-type HIV-1 Nef and the other samples.

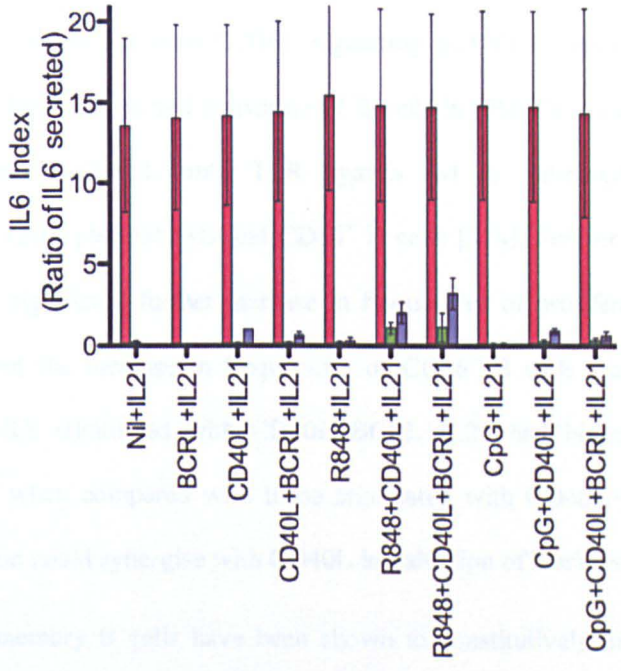

Figure 6.23: IL6 indices after pre-incubation with HIV proteins and subsequent exposure to various stimulant-combinations. All samples were pre-incubated with recombinant HIV-1 Nef (red), recombinant mutant (non-myristoylated) HIV-1 Nef (Green) or media (Blue). Data represents four independent experiments. Bars show mean ±SEM.

Stimulant	Mean(standard error) IL6 index			P value		
	Nef	MNef	Nil	Nef vs MNef	Nef vs Nil	MNef vs Nil
NIL+IL21	13.55(5.39)	0.22(0.10)	0.07(0.01)	0.0913	0.0873	0.2524
BCRL+IL21	14.05(5.74)	0.07(0.02)	0.06(0.02)	0.0920	0.0920	0.2542
CD40L+IL21	14.22(5.60)	0.14(0.04)	1.00(0.00)	0.0854	0.0992	0.0002
CD40L+BCRL+IL21	14.46(5.61)	0.17(0.06)	0.66(0.18)	0.0823	0.0936	0.1070
R848+IL21	15.47(5.90)	0.19(0.11)	0.30(0.22)	0.0785	0.0774	0.4022
R848+CD40L+IL21	14.84(5.98)	1.08(0.37)	2.03(0.64)	0.0932	0.1072	0.2369
R848+CD40L+BCR+IL21	14.75(5.76)	1.12(0.88)	3.19(0.97)	0.0733	0.1074	0.0035
CpG+IL21	14.82(5.86)	0.12(0.06)	0.11(0.06)	0.0850	0.0850	0.6277
CpG +CD40L+IL21	14.75(5.92)	0.26(0.12)	0.90(0.16)	0.0893	0.1006	0.0648
CpG+CD40L+BCRL+IL21	14.35(6.48)	0.32(0.17)	0.60(0.27)	0.1132	0.1135	0.1378

Table 6.8: Mean (and standard error of mean) of IL6 indices for the various stimulant-conditions after pre-incubation with wild-type HIV-1 Nef (Nef), mutant non-myristoylated HIV-1 Nef (MNef) or media (Nil) and the corresponding P values after pair-wise comparisons. Paired Student's T test was used. Significant P values are shown in red text.

6.3 Discussion

TLR signalling synergised with CD40L signalling and BCR signalling in inducing proliferation, differentiation and activation of B cells in PBMCs. Similar to a previous report, addition of CD40L onto TLR ligands led to increased frequencies of proliferating B cells, plasmablasts and $CD86^+$ B cells [328]. Further addition of BCR ligand led to a significant further increase in frequencies of proliferating B cells and plasmablasts, but the increase in frequencies of $CD86^+$ B cells was not significant. However, PBMCs stimulated with CD40L+BCRL+IL21 had higher frequencies of $CD86^+$ B cells when compared with those stimulated with CD40L+IL21, suggesting that BCR ligation could synergise with CD40L in induction of markers of activation.

While human memory B cells have been shown to constitutively express TLR7 and TLR9, naïve human B cells express TLR9 only after BCR stimulation [198]. Furthermore, TLR ligation has been shown to induce up-regulation of CD40 and IL21 receptor [209, 329]. These interactions provide a possible explanation to the observed synergy between BCR ligand, CD40L and TLR ligands in the induction of proliferation, activation and differentiation of B cells in the presence of IL21 in the current study.

Interestingly, adding BCR ligand to the stimulant-combinations, though effective in inducing differentiation into plasmablasts and proliferation, reduced antibody production. This phenomenon has been reported elsewhere [330-332]. Indeed, BCR ligation alone induces apoptosis. However, having CD40L or TLR signalling in addition to the BCR ligation rescues the B cells from apoptosis. The pro-apoptotic effect of BCR-only ligation is thought to be a mechanism to protect against auto-reactive antibody responses that may be induced by auto-antigens in the absence of other B-cell signals [333, 334]. Whether the blunting of antibody responses by BCR ligation in presence of CD40L and TLR signalling was due to its pro-apoptotic

effects is unclear. Even though the TLR and CD40 ligands are expected to rescue the cells from such BCR-induced apoptosis, their signalling might not have been strong enough to counteract the magnitude of BCR-induced apoptosis in the cultures. Alternatively, stimulation with BCR ligands in absence of TLR ligands or CD40 ligand may drive the B cells to preferentially differentiate into plasmablasts with little clonal expansion and little generation of memory B cells, processes that are favoured by TLR and CD40 signalling. Such an effect could lead to reduced antibody response in the long run in BCR stimulated cultures when compared with cultures that contain CD40L and TLR ligands without BCR ligand.

The TLR ligands R848 and CpG did not induce significant secretion of TNF-alpha and IL6 by PBMCs. However, addition of CD40L significantly increased the secretion of the two cytokines. Notably, further addition of BCR ligand did not cause further increment in secreted cytokines. Therefore, CD40 ligation seemed to be the only factor that significantly influenced secretion of TNF-alpha and IL6 by the PBMCs. It would be difficult to ascertain the cellular source of the cytokines, since both human and mouse B cells and monocytes have previously been shown to secrete TNF-alpha and IL6 upon stimulation with TLR ligands and CD40L [321, 324]. In fact, TLR ligation has been shown to synergize with CD40 ligation and BCR ligation in inducing the secretion of the two cytokines by human B cells [322]. It was therefore surprising to see that BCR ligation and TLR ligation did not significantly affect the secretion of the two cytokines in the current study, probably because PBMCs were used while the previous studies used purified B cells or purified monocytes. Differences in concentrations of TLR ligands and BCR ligands could have also played a role in the differences. Furthermore, considering that secretion of pro-inflammatory cytokines is very sensitive to endotoxin, the purity of the recombinant stimulants used in this study could have influenced the

secretion of TNF-alpha and IL6, though such purity was not determined.

Even though a previous study showed that recombinant HIV-1 Nef inhibited CD40L-mediated class switching of naive B cells [25], the results of the current study do not show any effect of the protein on proliferation, differentiation (plasmablasts and secretion of antibodies into culture supernatants) and activation (CD86 expression) of B cells upon stimulation with various combinations of CD40L, TLR ligands, BCR ligand and IL21. However, pre-incubation with wild-type HIV-1 Nef affected the secretion of TNF-alpha after exposure to the same stimulants.

According to the previous study, exposure of the B cells to the recombinant HIV-1 Nef inefficiently activated the NFκB pathway, leading to activation of negative feedback pathways that rendered the B cells refractory to subsequent NFκB pathway-dependent activation with CD40L+IL4 [25]. Since TLR9 and TLR7 too signal via NFκB, recombinant HIV-1 Nef would be expected to also inhibit the effect of R848 (TLR7 ligand) and CpG (TLR9 ligand) on B cells in the current study. Several differences in the experimental set-up could explain the differences in results between the two studies with regard to B-cell proliferation, differentiation and activation. Firstly, the current study involved the use of PBMCs while the previous study used purified naïve B cells. Consequently, there could have been some indirect effect of HIV-1 Nef via other immune cells in the PBMCs culture. Indeed, as discussed in the previous paragraph and in the literature review, HIV-1 Nef has numerous effects on monocytes and T cells [21, 22, 133, 309-311]. In addition to the secretion of IL6 and TNF-alpha, it induces secretion of other soluble factors that could affect B cells. For instance, Nef expressing monocytes secrete ferritin that in turn induces polyclonal activation of B cells [133]. Moreover, the monocyte-derived TNF-alpha and IL6 could potentiate the effect of other B-cell stimulants [323, 335].

Differences in the source and quality of the reagents could also have contributed to the differences in the results of the two studies. For instance, the previous study used trimeric CD40L from Immunex whereas the current study used a kit containing monomeric CD40L+enhancer for ligands (a reagent that cross links the CD40L to enhance its potency) from Enzo Life sciences [25]. Also, even though the identities and sources of the recombinant HIV-1 proteins used were similar, differences in handling and storage conditions could have affected their integrity. However, wild-type HIV-1 Nef in the current study induced secretion of IL6 and TNF-alpha in PBMCs in agreement with a previous study on monocytes, suggesting that it was not defective [310].

Finally, the previous study focused on the effects of HIV-1 Nef on class switching of naïve B cells while the current study evaluated effects on proliferation, differentiation and activation of B cells. Even though all these processes rely on the NFκB pathway, their other differences could have led to the observed differences in the results.

Chapter 7 Summary and conclusion

The effect of HIV on the B-cell compartment in adults has been well characterized. Active viraemia has been shown to cause aberrant polyclonal activation of B cells and expansion of certain B-cell subsets that are normally in low frequencies in normal uninfected adults [84, 123, 215, 226, 228, 229]. The function of B cells in highly viraemic HIV-infected adults has also been shown to be compromised, with such adults having low levels of vaccine-specific antibodies and low frequencies of vaccine-specific memory B cells [84, 229, 243, 244, 253]. Children differ from adults in that their immune system is relatively immature. Furthermore, their B-cell compartment develops in a background of HIV infection, unlike adults who get infected later in life when their immune system is already mature. The interaction between HIV and the B-cell compartment may therefore differ in children when compared with adults. However, the phenotype and function of B cells in HIV-infected children has not been studied to the same extent as in adults.

The overall goal of this study was to determine the B-cell defects and their causes in HIV-infected children. In particular, I aimed to:

1. Describe the subset distribution of B cells in children infected with HIV.
2. Describe the B-cell responses to common childhood vaccines in children infected with HIV i.e.
    a. Quantities of antibodies in circulation.
    b. Avidities of antibodies in circulation.
    c. Frequencies of memory B cells in peripheral blood.
3. Describe the memory B-cell responses to pneumococcal capsular polysaccharides in children after natural exposure or vaccination with a pneumococcal conjugate vaccine and compare those responses with natural and vaccine-induced anti-pneumococcal responses in HIV-infected children from HIV clinic and community control children from Ngerenya cohort.

4. Determine the in vitro response of B cells to different B-cell stimulant-combinations and evaluate the in vitro effects of recombinant HIV-1 Nef protein on B-cell responses to such stimulant-combinations.

For the assessment of phenotypes and function of B cells in HIV-infected children, a cross-sectional study was done at the HIV clinic (CCRC). These HIV-infected children were then compared with community control children from the Ngerenya malaria cohort. For determination of memory B-cell responses to pneumococcal capsular polysaccharides in children before and after vaccination with a pneumococcal conjugate vaccine, children were recruited into a larger clinical trial that evaluated immunogenicity and reactogenicity to a PHiD-CV vaccine in children aged 12-59 months. Memory B-cell responses were evaluated in a subset of the children aged 12-24 months at enrollment and at 30 days following each of the two doses of the vaccine. The responses were then compared to those observed in HIV-infected CCRC children and community control Ngerenya children. In vitro experiments were done using lymphocytes from buffy coats that had been collected from healthy donors. B-cell responses were assessed after stimulating the lymphocytes with various stimuli. Effects of HIV-1 Nef on the B-cell responses to the stimuli were also assessed after pre-incubating the lymphocytes with wild-type HIV-1 Nef, mutant non-myristoylated HIV-1 Nef and media.

Flow cytometry was used for determination of the expression of surface markers on B cells. The surface markers were then used to identify the various B-cell subsets. Frequencies of each B-cell subset were compared between different groups of HIV-infected children and community control children. Flow cytometry was also used for determination of proliferation, differentiation and activation of lymphocytes after culture in the in vitro experiments. Enzyme Linked Immunoassay (ELISA) was used

to determine the levels of antibodies against vaccine antigens and pneumococcal capsular polysaccharides. The same assay was used to determine levels of total immunoglobulins in culture supernatants. A modified ELISA was used to determine the avidity indices of antibodies against their cognate antigens. Commercial cytokine-ELISA kits were used in accordance to manufacturers' instructions to determine levels of TNF-alpha and IL6 in culture supernatants. Cultured B-cell ELISpots were done to determine the frequencies of antigen-specific memory B cells.

Similar to previous studies in adults, in this study, the frequencies of activated B-cell subsets (i.e. activated mature B cells, atypical memory B cells and plasmablsts) were higher in the high viraemia HIV-infected children when compared with community controls and low viraemia HIV-infected children, suggesting that HIV viraemia was the driving force in their expansion [84]. A similar observation was also observed in children in a previous study, though the three activated subsets were not differentiated [234]. In the generalized quantile regression model, viral load was the common predictor of frequencies of all activated subsets. $CD4^+$ T-cell percentages significantly predicted frequencies of atypical memory B cells only. On the other hand, frequencies of resting memory B cells were lower in the entire HIV-infected cohort when compared with community controls. This has been shown to be the case in adults too and has also been reported in some studies on children [228, 230, 231, 234]. Notably, unlike previous reports on adults, there were no differences in immature transitional B cells in the different groups of children in the current study [226].

Interestingly, there was a statistical interaction between level of viraemia and age with regard to age-related acquisition of resting memory B cells. Similar to community controls, the groups of children that had low viraemia showed age-related increase in frequencies of resting memory B cells while those groups that had high viraemia did not show the same. This observation has never been reported elsewhere and provides a

good reason to consider if all children, regardless of their age, should be put on HAART as soon as they are diagnosed with HIV. Currently, according to the WHO recommendations, all children who are diagnosed with HIV in the first two years of life are to be put on HAART immediately. Until recently, children who are diagnosed with HIV after their second birthday and before 5 years of age were to be put on HAART only if their $CD4^+$ T-cell counts fell below 750 cells/μL or below 25% or if they were in WHO clinical stage 3 or 4 while children who were above 5 years were to be put on HAART only if their $CD4^+$ T-cell counts fell below 350 cells/μL or if they were in WHO clinical stage 3 or 4 [80]. A recent revision of the WHO guidelines now recommends all children below five years of age to be put on HAART the moment they are diagnosed with HIV, but scientific evidence to support that recommendation is lacking [76]. Furthermore, the new WHO recommendations are yet to be adopted into the national treatment guidelines for the various countries. Considering that 90% of the HIV-infected children live in the resource-poor Sub-Saharan Africa where only 28% of eligible children were receiving HAART as at the year 2011 [45], many children could be missing the opportunity of being started on HAART early. In addition, delivery of babies at home is still common in some parts of the region [336, 337], suggesting that a significant number of children continue to miss out on PMTCT services and early initiation of HAART if they are infected. Such children, if they live long enough, are likely to be diagnosed with HIV late and are put on HAART if their $CD4^+$ T-cell percentages/counts deteriorate to a certain level in accordance with their country's policies. According to this study, high viraemia continues to take a toll on their immune system by denying them a chance to accumulate memory B cells with age. Control of viraemia by putting all HIV-infected children on HAART may enable them to grow immunologically like healthy children.

Similar to previous studies in adults and children, the HIV-infected children had

lower levels of antibodies against childhood vaccines when compared with community controls [84, 229, 243-245, 257-264, 266-268]. The HIV-infected children in the current study also had lower avidity indices of IgG against the same vaccine antigens and lower frequencies of memory B cells against some vaccine antigens when compared with community control children. This confirms the few studies that have assessed the effect of pediatric HIV on antibody avidity maturation and frequencies of antigen-specific memory B cells [232, 245, 261]. However, no differences were observed between the two groups of children with regard to naturally acquired responses against pneumococcal capsular polysaccharides. This suggests that HIV-infected children make sub-optimal B-cell responses to vaccines at the time of immunization or experience depletion of the responses that they make, raising the question of whether they should be revaccinated after being put on HAART. At the moment, there are no programs in place for revaccination of HIV-infected children. The current study, together with many other previous reports, suggests that revaccination schedules could be beneficial to these children [338].

Memory B-cell frequencies were also assessed against pneumococcal capsular polysaccharides from serotypes 19F, 14, 23F, 6B and 1 among healthy children who took part in a clinical trial for a 10-valent Pneumococcal non-typable *Haemophilus Influenza* protein D conjugate vaccine (PHiD-CV). Memory B cells against tetanus toxoid and diphtheria toxoid were also evaluated in the same children. The two proteins had been used to conjugate some of the polysaccharides in the vaccine. Vaccination of healthy children with two doses of PHiD-CV elicited significant increase in frequencies memory B cells against all capsular polysaccharides with the exception of serotype 6B. There was also a significant increase in frequencies of memory B cells against the conjugating proteins, i.e. tetanus toxoid and diphtheria toxoid, upon vaccination with

PHiD-CV. Similar effects were observed in community control children who received the PHiD-CV as part of the catch-up campaign in the community. Notably, children who had serotype 19F carriage at enrollment elicited poorer responses against serotype 19F after vaccination. Importantly, older HIV-infected children at the HIV clinic were comparable to younger healthy children in the trial with regard to anti-pneumococcal responses, suggesting that HIV stunts natural acquisition of memory B cells against pneumococcal capsular polysaccharides.

The in vitro response of B cells in PBMCs after exposure to various combinations of B-cell stimulants was assessed in a bid to set up a system for evaluating the effect of recombinant HIV proteins on B cells. Similar to previous reports, synergistic effects were observed between BCR signalling, TLR signalling and CD40 signalling with regard to proliferation, differentiation into plasmablasts and expression of activation markers [328, 330]. Synergy was also observed between TLR signalling and CD40 signalling with regard to secretion of immunoglobulins. However, BCR signalling dampened the secretion of immunoglobulins, a phenomenon that has been reported elsewhere [330]. Secretion of TNF-alpha and IL6 was dependent on CD40 signalling. When the effect of HIV-1 Nef was assessed on PBMCs stimulated with the different stimulant-combinations, unlike a previous report that suggested HIV-1 Nef could inhibit class switching of B cells, there was no observed effect on proliferation, differentiation into plasmablasts, expression of activation markers and secretion of immunoglobulins in the B-cell compartment [25]. However, wild-type HIV-1 Nef induced secretion of TNF-alpha by PBMCs, similar to other studies on monocytes [310]. Differences in cytokine responses between PBMCs and purified naïve B cells upon exposure to HIV-1 Nef could explain the differences in results between the current study and the previous study.

Future work on B cells in HIV patients would entail the determination of the mechanisms of the observed defects. Of particular interest is the cause of polyclonal activation, whose causes and consequences have not been fully elucidated. Even though gp120 has been shown to activate B cells polyclonally via DC-SIGN and the B-cell receptor, the possible role of single stranded RNA via toll like receptor 7 has not been studied. Furthermore, polyclonal B-cell activation could be an immune evasion strategy whereby the virus diverts the energies of the immune system towards responses that are not relevant for the invading pathogen. For instance, activated B cells of other specificities could compete with the HIV-specific B cells in utilization of T-cell help, a phenomenon that could have serious effect in blunting the antibody response against HIV.

It would be also desirable to do the in-vitro experiments using purified B cells to check if the effects of recombinant Nef differ from those observed in my work.

With the PHiD-CV vaccine already rolled out for routine childhood immunisation, it would be practical and desirable to study further the memory B-cell responses in healthy and HIV-infected children who receive it at 6 weeks, 10 weeks and 14 weeks; memory B-cell responses against pneumococcal antigens have not been evaluated in children who receive PHiD-CV at those ages.

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Appendices

Ethical approvals

KENYA MEDICAL RESEARCH INSTITUTE

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DIRECTOR
CENTRE FOR GEOGRAPHIC MEDICINE RESEARCH, COAST

KEMRI/RES/7/3/1 November 8, 2009

TO: DR. BRITTA URBAN (PRINCIPAL INVESTIGATOR)

THROUGH: DR. NORBERT PESHU,
THE DIRECTOR, CGMR-C
KILIFI

RE: SSC PROTOCOL NO. 1633 (*RE-SUBMISSION*): PHENOTYPE AND FUNCTION OF B CELL IN CHILDREN INFECTED WITH THE HUMAN IMMUNODEFICIENCY VIRUS

Make reference to your letter dated October 5, 2009. Thank you for your response to the issues raised by the Committee. This is to inform you that the issues raised during the 168th meeting of KEMRI/National Ethics Review Committee held on Tuesday 14th July 2009, have been adequately addressed.

It is now clear that the information we require from the healthy children for the purpose of comparison with values in children infected with HIV is already available as part of Dr. Nduati's PhD awarded in June 2009 by the University of Dundee and other data that will be generated in May 2010 as part of the ongoing investigations into B cell responses during and after acute malaria collected under SSC 1131.

Requesting for permission to use data generated under SSC Protocol 1131 for comparison with data generated in children infected with HIV in the above mentioned protocol, is really beyond the purview of this committee. Use of another study's data should be sorted out between the principal investigators of the studies in question.

Due consideration has been given to ethical issues and the study is hereby granted approval for implementation effective this **8th day of November 2009,** for a period of twelve (12) months.

Please note that authorization to conduct this study will automatically expire on **7th November 2010.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **26th September 2010**.

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the ERC prior to initiation. You may embark on the study.

Yours sincerely,

R. C. KITHINJI,
FOR: SECRETARY,
KEMRI/NATIONAL ETHICS REVIEW COMMITTEE

KEMRI / WELLCOME TRUST RESEARCH PROGRAMME
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KEMRI/RES/7/3/1 **21ST MAY, 2013**

TO: **BITTA URBAN,**
(PRINCIPAL INVESTIGATOR)

THROUGH: **DR. SABAH OMAR,**
DIRECTOR, CGMRC,
KILIFI

DIRECTOR CENTRE FOR GEOGRAPHIC MEDICINE COAST 29/5/13

RE: SSC PROTOCOL 1633 (*CONTINUING REVIEW REPORT*) PHENOTYPE AND FUNCTION OF B CELLS IN CHILDREN INFECTED WITH HUMAN IMMUNODEFICIENCY VIRUS.

This is to inform that during the 215th meeting of the KEMRI/ERC meeting held on **May 21st 2013**, the Committee **conducted the annual review and approved** the above referenced application for another year.

This approval is valid from today **May 21st, 2013** through to **May 20th, 2014.** Please note that authorization to conduct this study will automatically expire on **May 19th, 2014.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the **ERC** secretariat by **April 9th 2014.**

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the SSC and ERC for review prior to initiation.

Yours sincerely,

EAB

DR. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

KEMRI/WT RESEARCH TRUST 29 MAY 2013 RECE P. O. Box 2... KILIFI

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KEMRI/RES/7/3/1 **September 9, 2009**

TO: **DR. LAURA HAMMITT (PRINCIPAL INVESTIGATOR)**

THROUGH: **DR. NORBERT PESHU,**
THE DIRECTOR, CGMR-C
KILIFI

Funds Granted 22/09/09

RE: **SSC PROTOCOL NO. 1635 (*ETHICS REVIEW*): IMMUNOGENICITY AND REACTOGENICITY OF 10-VALENT PNEUMOCOCCAL CONJUGATE VACCINE IN CHILDREN AGED 12-59 MONTHS.**

This is to inform you that during the 169th meeting of KEMRI/National Ethics Review Committee held on Tuesday 1st September 2009, the abovementioned study was reviewed.

Due consideration has been given to ethical issues and the study is hereby granted approval for implementation effective this **9th day of September 2009,** for a period of twelve (12) months.

Please note that authorization to conduct this study will automatically expire on **9th September 2010.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **29th July 2010.**

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the ERC prior to initiation. You may embark on the study.

Yours sincerely,

R.C.Kithinji

R. C. KITHINJI,
FOR: SECRETARY,
KEMRI/NATIONAL ETHICS REVIEW COMMITTEE

KEMRI / WELLCOME TRUST
RESEARCH PROGRAMME
15 SEP 2009
RECEIVED
P. O. Box 230-80108 KILIFI

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200 NAIROBI, Kenya
Tel: +254 (020) 2722541, 2713349, 0722-205901, 0733-400003, Fax +254 (020) 2720030,
E-mail: director@kemri.org; info@kemri.org Website: www.kemri.org

KEMRI/RES/7/3/1 **25th Aug 2006**

Dr. K. Marsh
CGMR-C,
KILIFI.

Thro'
Director,
CGMR-C,
KILIFI.

Forwarded: 31/08
For:

Dear Sir,

Re: SSC Protocol No. 1131 (Revised) – **Integrated studies of the development of natural immunity to malaria in children in Kilifi district, by K Marsh *et al***

During the 136th Meeting of the KEMRI/National Ethical Review Committee held on the 22nd of August 2006 the above protocol was discussed.

Thank you for a well written and informative proposal that aims to establish an epidemiological framework in which to carry out longitudinal studies of the development of immune responses to malaria.

Due consideration has been given to ethical issues and therefore the protocol is granted approval. You are responsible for reporting to the Ethical Review Committee any changes to the protocol or in the Informed Consent Document. This includes changes to research design or procedures that could introduce new or more than minimum risk to human subjects. In addition it is requirement of this Committee to include the contact of the KEMRI/NERC which is Secretary, KEMRI/NERC, +254-02-2722541.

Kindly remit a copy of the revised protocol for our records.

R.C.M. Kithinji
For: Secretary,
KEMRI/National Ethical Review Committee

Informed consent form for recruiting children at CCRC

KEMRI – Wellcome Programme Information Sheet and Consent Form

Study title: Phenotype and function of B cells in children infected with the human immunodeficiency virus-1 (HIV-1)

Lay title: Type and function of B cells (immune system cells) in children infected with the human immunodeficiency virus-1 (HIV-1)

Institution	Individuals
KEMRI CGMRC	**Britta Urban (Principal Investigator), Evelyn Gitau, Eunice Nduati, Pete Bull, Francis Ndungu, Jay Berkley, Hellen Nabwera, Maureen Mutinda**

Your child's illness/symptoms

Your child is attending the Comprehensive Care and Research Clinic (CCRC) where care is given for people infected with HIV. The normal standard of care for people infected with HIV is monthly clinical check-ups and checks for the body's cells, which protect against infection - CD4 counts. In addition to these tests a drug called Cotrimoxazole, which prevents infection by other diseases is given to patients, as well as Anti-retroviral treatment (ART), which reduces the amount of HIV in the body. These drugs are given according to national recommendations.

What is KEMRI and what is this study about?

KEMRI is a government organization that carries out medical research to find better ways of preventing and treating illness in the future for everybody's benefit. One illness KEMRI is currently trying to learn more about is infection with HIV. There is currently very little information in Kenya about how children infected with HIV fight

common childhood infections such as malaria or how they respond to vaccines. To help us learn more we are asking a total of 300 children infected with HIV to participate in a study on how HIV affects the defense system of the body. We would like to include 100 children before they start cotrimoxazole, 100 children who already receive co-trimoxazole and 100 children who already receive ART. We will compare the function of the body's defense system in children infected with HIV to the function of the body's defense system to children not infected with HIV.

What will it involve for me/my child if I agree?

We will use a small amount of the blood that will be taken as part of your child's normal treatment at the CCRC for this research. The extra amount taken for research will be less than 1 tsp (3ml). We will take this sample only once when your child has a small amount of blood drawn for routine clinical care.

The rest of your child's treatment will be according to the Ministry of Health guidelines. If there are any other research activities that KEMRI staff would like your child to participate in, staff shall explain and ask your permission first.

Are there any risks or advantages to me or my c hild of participating?

Our priority for every patient is his/her care.

Taking blood from the arm causes a small amount of pain, but the amount taken is too small to affect your child's health.

There is no direct benefit to your child, The information gained from this research may help us to improve the care of children who have HIV in the future. Where results could be helpful for your child's treatment, for instance how much virus is in your child's body, these will be given to the doctor or clinical officer providing your child's care.

What happens if I refuse to participate?

All participation in research is voluntary. You are free to decide if you want your child to take part. Your child will have the same level of care whether they take part or

not. If you do agree you can change your mind at any time and withdraw your child from the research. This will not affect your child's care now or in the future.

What happens to the samples?

Individual names are removed from all samples and replaced by codes, to ensure that samples can only be linked to the participants by people closely concerned with the research.

Most of the research tests that will be done on the blood will be done here in Kilifi. We will test in the laboratory how infection with HIV alters your child's ability to fight infection and respond to vaccines. Some tests will provide information on how well you child is coping with the HIV virus i.e. how much virus is in the blood. We will give the results of these tests to the Clinician looking after your child as soon as they become available. Some test that cannot be done in Kenya, part of the samples will be sent to our collaborators in Sweden.

After the research, a small portion of the blood will be stored. In the future, new research about HIV or malaria may be done on these samples. Future research must first be approved by a national independent expert committee in Nairobi to ensure participants' safety and rights are respected.

Who will have access to information about me/my child in this research?

All our research records are stored securely in locked cabinets and password protected computers. Only a few people who are closely concerned with the research will be able to view information from participants.

Who has allowed this research to take place?

An independent national committee and a committee in Kilifi have looked carefully at this work and agreed that the research is important, that it will be conducted properly and participants' safety and rights have been respected.

What if I have any questions?

You may ask any of our staff questions at any time. You can also contact those who are responsible for the care of your child and this research:

PI's name(s) and contacts

Dr. Britta Urban

KEMRI- Wellcome Trust,

P.O.Box. 230, Kenya. Telephone: 0728024743 or 041 7522 063

If you want to ask someone independent anything about this research please contact

Community Liaison Manager, KEMRI – Wellcome Trust

P.O.Box 230, Kilifi. Telephone: 0723342780 or 041 7522 063

Or

The Secretary - KEMRI/National Ethics Review Committee

P. O. BOX 54840-00200, Nairobi, Tel number: 020 272 2541 Mobile: 0722205901 or 0733400003

KEMRI-Well come Research Programme Consent form for sample only studies

Study title: Phenotype and function of B cells in children infected with the human immunodeficiency virus-1 (HIV-1)

I, being a parent/guardian of _____ (name of child), have had the research explained to me. I have understood all that has been read and had my questions answered satisfactorily. I understand that I can change my mind at any stage and it will not affect me/my child in any way.

Please tick the boxes below where relevant:

☐ **I agree to allow my child to take part in this research**

☐ **I agree to samples being stored**

☐ **I agree to samples being exported**

Subject/Parent/guardian's signature: _____ **Date** _____

Subject/Parent/guardian's name: _____ **Time** _____

(Please print name)

I certify that I have followed all the study specific procedures in the SOP for obtaining informed consent.

Designee/investigator's signature: _____ **Date** _____

Designee/investigator's name: _____ **Time** _____

(Please print name)

Only necessary if the parent/guardian cannot read:

I *attest that the information concerning this research was accurately explained to and apparently understood by the parent/guardian and that informed consent was freely given by the parent/guardian.

Witness' signature: _____ **Date** _____

Witness' name: _____ **Time** _____

(Please print name)

***A witness is a person who is independent from the trial or a member of staff who was not involved in gaining the consent.**

Thumbprint of the parent as named above if they cannot write:

THE SUBJECT/PARENT/GUARDIAN SHOULD NOW BE GIVEN A SIGNED COPY TO KEEP.

Case report form for recruiting children at CCRC

Phenotype and Function of B Cells in Children Infected With HIV

Arm..... B cell Study ID..... CCRC No.....Today's Date.....

1. DOB .....Gender ..... Muac..... .....
2. Date of Enrollment into CCRC.....
3. Date of Diagnosis and Test Used
   - ☐ PCR Date.....
   - ☐ Antibody Test Date.....
4. Feeding practice
   - ☐ 0-6months
     a) Exclusive Breasting Feeding
     b) Mixed Feeding
     c) No Breastfeeding
   - ☐ 6 and above
     a) Exclusive Breast Feeding
     b) Mixed Feeding
     c) No Breastfeeding
5. Therapy History
   - ☐ No previous Therapy
   - ☐ CTX alone
   - ☐ ARV Alone
   - ☐ Both CTX & ARV
6. Date started therapy
   - ☐ CTX .....
   - ☐ ARV .....
7. Name of ARV Therapy
   - ☐ 1st Line.....
   - ☐ 2nd Line .....
8. Adherence
   - ☐ Satisfactory
   - ☐ Unsatisfactory
   - ☐ Stopped
   - ☐ Not Applicable
9. DPT Vaccination given ☐ Yes ☐ No
10. Pneumococcal vaccination given ☐ ☐
11. Measles..... ☐ ☐
12. Consent Given
    - ☐ Yes
    - ☐ No

Form Filled by.....

List of reagents

Reagent	Supplier	Catalogue number
Affinipure F(ab')2 fragment goat antihuman IgA+IgG+IgM	Jackson Immunoresearch	109-006-064
Anti-human CD21 PE	eBioscience	12-0219
Anti-human IgD PE	Beckman coulter	736000
Anti-mouse Ig, k/negative control (FBS) compensation particles set	BD	552843
AP conjugated affinipure goat anti-human IgG, Fcγ fragment specific	Jackson Immunoresearch	109-055-098
AP conjugated affinipure goat anti-human IgM	Jackson Immunoresearch	109-055-043
APC mouse anti-human CD21	BD	559867
APC-H7 mouse anti-human CD20	BD	560734
Arc amine reactive compensation bead kit	Invitrogen	A10346
Beta-mercapto ethanol	Gibco	31350010
Brilliant Violet 421 anti-human CD19	Biolegend	302234
CD10 FITC	BD	332775
CD19-ECD	Beckman coulter	A07770
CD210FITC	Beckman coulter	IM0473U
CD27-PC5	Beckman coulter	6607107
CD3 ECD	Beckman coulter	A07748
CD3 FITC	BD	345763
CD4 PE-Cy7	BD	348809
CD40L, soluble (human)(recombinant)set	Enzo Life sciences	ALX-850-064
CD8 APC-H7	BD	641400
CD86-PE	Biolegend	305406
Celltrace CFSE cell proliferation kit	Invitrogen	C34554
CpG-ODN 2006	Hycult Biotech	HC4039
Diphtheria toxoid	Statens Serum Institute	
Goat antihuman Ig's(Polyvalent)	Invitrogen	H17000
Guanidine hydrochloride	Sigma	G3272
HEPES buffer	Sigma	H0887
HIV-1 Nef protein (G2A)His, non-myristoylated	Jena bioscience	PR-383
HIV-1 Nef protein (wild type)His, myristoylated	Jena bioscience	PR-382
Human and mouse CpG-B DNA	Hycult biotech	HC4039
IgG human serum	Sigma	I2511
IgM human serum	Sigma	I8260
IL6 Quantikine ELISA kit	R & D	D6050
Live/dead fixable aqua dead cell stain kit	Life technologies	L34957
Lymphoprep	Axis-Shield	1114544

Measles (Rubeola)virus antigen	Meridian Life Science	7604
methulated human serum albumin	NIBSC	99/592
Newborne bovine serum	Sigma	N4637
OPD peroxidase substrate	Sigma	P9187
Optilyce C lysis solution	Beckman coulter	A11895
OPtilyce C Lysis solution	beckman coulter	A11895
PE anti-human CD10	Biolegend	312203
PE rat anti-human CD267 (TACI)	BD	558414
PE/Cy7 anti-human CD38	Biolegend	303515
Penicillin streptomycin solution	Sigma	P7539
Peroxidase conjugated antihuman IgM	Jackson Immunoresearch	709-036-073
Peroxidase-conjugated affinipure F(ab')2 Fragment donkey antihuman IgG	Jackson Immunoresearch	709-036-098
pneumococcal capsular polysaccharides	ATCC	
R848	Invivogen	tlrl-r848
Recombinant human IL21	Peprotech	AF-200-21
RPMI-1640 medium	Sigma	R0883
Staphylococcus aureus Cowan strain	Sigma	P 7155
Tetanus toxoid	Statens Serum Institute	
TNF-alpha Quantikine ELISA Kit	R & D	DTA00C